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(57) Abstract

The N-terminal region of 23 amino acids of ODV-E66 is composed predominantly of hydrophobic residues and was shown to be necessary and sufficient to target a cytoplasmic marker protein, e.g., beta-galactosidase, green fluorescent protein and URF13, to the intranuclear viral-induced unit-membranes and viral envelopes. The 23 hydrophobic amino acid sequence is (I).

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NUCLEOTIDE AND AMINO ACID SEQUENCE AND USES THEREOF

5   Background of the Invention

This invention is directed to a nucleotide and amino acid sequence that serves to target foreign proteins to the occlusion derived virus (ODV) envelope, e.g., the N-terminal hydrophobic twenty three (23) amino acid sequence derived from ODV-E66.

10 I.   INTRODUCTION

Naturally occurring insect baculoviruses have received considerable attention for their potential to be used as non-chemical, environmentally safe control agents for insect pests. They infect only arthropods, and each virus strain infects only one or a
15 limited number of species. Many of the virus hosts are commercially important agricultural insect pests. Examples include infestation of cotton, sorghum and corn by the bollworm/budworm complex, and infestation of garden crops by the cabbage looper and tomato hornworm. As important, beneficial insects that are natural
20 predators of insect pests are not hosts for baculoviruses. Baculovirus AcMNPV has undergone 5 controlled release trials and these trials indicate that when the virus is genetically modified by deletion of a single gene, it does not persist in the environment

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(Cory and Bishop, 1995). Thus, genetically engineered baculoviruses can be used safely as a "one pass" insecticide that will kill the insect pest and not persist in the environment.

    The host range of insect baculoviruses has been extensively
5 studied and no evidence of infection or pathogenic responses have been identified in non-host insects, plants, vertebrates and man (Groner, 1986). This feature may make baculoviruses as ideal agent to be modified and used for the delivery of drugs, genes, or therapeutics. Both the use of baculoviruses as insecticides and as
10 treatment delivery agents in animals and human diseases are discussed, using an amino acid sequence which targets proteins to the viral envelope of the occlusion derived virus (ODV).

    ODV is the primary infectious agent to the insect host. The ODV envelope fuses with the plasma membrane of the gut cell, thus
15 releasing the nucleocapsid and viral DNA into the cell (Kawanishi et al., 1972). In this process, viral envelope proteins are incorporated into the gut cell plasma membrane.

A.   General Properties of the Baculovirus

    The family Baculoviridae consists of viruses with an
20 enveloped rod-shaped virion containing a circular double-stranded DNA genome ranging from 88 to 153 kb (Murphy et al., 1995). Baculoviruses have been isolated only from arthropods; primarily from insects of the order Lepidoptera, but also from Hymenoptera, Diptera, Coleoptera, Neuroptera, Thysanura, and Trichoptera as well as from
25 the crustacean order Decapoda (shrimp). Individual baculovirus may infect one species or multiple species within the same order (e.g., AcMNPV infection of Lepidopteran). However, none of the baculoviruses infects a large number of insects, or members of different Orders of the Class Insecta. Autographa californica
30 multinucleocapsid nuclear polyhedrosis virus (AcMNPV) is unusual among baculoviruses because it displays a wider host range than most baculoviruses, which includes 32 species in 12 families (Martignoni et al., 1982).

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AcMNPV is the most extensively studied baculovirus and its genome sequence is known (Ayres et al., 1994). It is distinguished by a unique biphasic life cycle in its lepidopteran host insect (reviewed in Blissard and Rohrmann, 1990). Infection produces high
5   titers of two forms of progeny virus, budded virus (BV) and ODV. Both forms are essential for natural propagation of the virus. Infection initiates when the insect feeds on a food source contaminated by viral occlusions. The alkaline midgut fluids dissolve the viral occlusion, and release ODV to infect the midgut
10 epithelium. Early in infection nucleocapsids assemble in the nucleus, migrate to the cell surface, and obtain an envelope by budding through the plasma membrane into the insect hemolymph. This form of virus is named BV and is responsible for secondary or systemic infection of a variety of tissues in the insect. Late in
15 infection the production of BV decreases. Instead of budding from the cell surface, the nucleocapsids remain within the nucleoplasm where they are enveloped and occluded within the viral occlusion. This form of virus is named occlusion derived virus or "ODV." The insect midgut epithelium is generally believed to be the site of
20 primary infection in vivo (Adams and McClintock, 1991).

ODV and BV contain the same genome, yet exhibit significant differences in morphology, timing and cellular site of maturation, structural proteins, source of viral envelopes, antigenicity, and infectivity (Blissard and Rohrmann, 1990; Braunagel and Summers,
25 1994). The differences between the envelopes of ODV and BV may function to regulate important aspects of the role of these two viral forms in infectivity, host range, and tissue specificity.

B.   Regulation of Viral Gene Expression

AcMNPV gene expression is temporally regulated. AcMNPV
30 genes are classified into two phases (early and late) which are further subdivided into four classes: immediate early or "IE", delayed early, late, and very late (Blissard and Rohrmann, 1990). Promoters of immediate early genes are active in uninfected insect

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cells and are expressed very early in infection, thus making them ideal promoters for the expression of proteins with insecticidal properties to compromise infected insects at a very early stage or for the expression of genes in transiently transfected or stably
5 transformed cells. Five immediate early (IE) genes have been identified, e.g., IE-0 (Chisholm and Henner, 1988) and IE-1 (Kovacs et al., 1991a, b; Choi and Guarino, 1995a, b, c), IE-2 (Carson et al., 1988, 1991), PE-38 (Krappa et al., 1992), and ME53 (Morsdorf et al., 1993). Very late genes are highly expressed very late during
10 infection and their promoters have been utilized to express a variety of proteins using the baculovirus expression vector system. Two very late genes are known: polyhedrin (Hooft van Iddekinge et al., 1983) and p10 (Kuzio et al., 1984). The identity and nucleotide sequence of promoters representing all classes of genes are known and can be
15 used for the expression of chimeric genes attached to them or simultaneous expression of multiple genes at controlled times.

    C.   Virion Structure

        1.   Nucleocapsid

        The differences in nucleocapsid protein composition between
20 ODV and BV of AcMNPV have been investigated (Braunagel and Summers, 1994). Several proteins have been identified as common nucleocapsid components in both phenotypes of AcMNPV (Blissard and Rohrmann, 1990; Rohrmann, 1992), including: p39 (Thiem and Miller, 1989); p80 (Lu and Carstens, 1992); p6.9 (Wilson et al., 1987; Wilson, 1988); p24
25 (Wolgamot et al., 1993). Homologs of these nucleocapsid proteins have also been identified in other baculoviruses.

        2.   Viral envelope

        Generally, the ODV envelope is closely associated with the nucleocapsid and has no apparent specialized morphology, whereas the
30 BV envelope is loose-fitting and has a specialized region (peplomer) at one end of the virion which is enriched with the viral envelope glycoprotein gp64 (Adams and McClintock, 1991).

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The biochemical composition of both viral envelopes is fairly well defined. A recent study shows major differences between BV and ODV in envelope protein SDS-PAGE profiles, major antigens, lipid, and fatty acid compositions (Braunagel and Summers, 1994).

5   a.   BV envelope proteins

So far, only one BV envelope protein (gp64) has been mapped in the genomes of AcMNPV (Whitford et al., 1989), OpMNPV: Orgyia pseudotsugata multinucleocapsid nuclear polyhedrosis virus (Blissard and Rohrmann, 1989), and CfMNPV: Christoneura fumiferana

10 multinucleocapsid nuclear polyhedrosis virus (Hill and Faulkner, 1994). Gp64 is a type I transmembrane protein which is N-glycosylated (Rohrmann, 1992).

AcMNPV encodes a ubiquitin-like protein which is integrated into the BV envelope through a novel type of phospholipid anchor

15 (Guarino et al., 1995).

The baculovirus apoptotic inhibitor p35 is also detected in purified BV by Western-blot analysis; however, the exact location of p35 in the virion is unknown. It is not known whether p35 is present in ODV (Hershberger et al., 1994).

20   b.   ODV envelope proteins

Several ODV envelope proteins of AcMNPV have been identified, including ODV-E66 (Hong et al., 1994), ODV-E56 (Braunagel et al., 1996a), and ODV-E18/E35 (Braunagel et al., 1996b). Other candidate ODV envelope proteins have also been identified. A fatty

25 acylated protein of 26.4 kDa (p26.4) is associated with AcMNPV ODV (Rohrmann, 1992). The p74 protein is known to play a role in ODV infectivity (Kuzio et al., 1989) and is believed to be a ODV structural protein; however, its location within the virus has not been determined. Two ODV envelope proteins have been identified in

30 other baculoviruses: Vp17 of Plodia interpunctella granulosis virus (Funk and Consigli, 1993) and ODV-E25 (or p25) of Orygia

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pseudotsugata multinucleocapsid nuclear polyhedrosis virus (Russell and Rohrmann, 1993).

3. Tegument

An ODV-specific structural O-linked glycoprotein, gp41, is
5   suggested to reside between the envelope and nucleocapsid, ie. the tegument (Whitford and Faulkner, 1992a, b).

D. Replication pathway of AcMNPV

The replication pathway of AcMNPV is briefly discussed below. A more extensive review can be found in Adams and McClintock
10 (1991).

1. Virion attachment and penetration

Two routes, adsorptive endocytosis (or viropexis) and direct fusion of BV envelope with plasma membrane, are initially proposed for entry of BV into cultured cells. Although BV may enter cells by
15 fusion (Volkman et al., 1986; Kozuma and Hukuhara, 1994), the majority of data indicates that the primary route is by adsorptive endocytosis (Charlton and Volkman, 1993).

By extrapolation with other enveloped animal viruses, especially orthomyxoviruses (White, 1990), a model is proposed to
20 explain the BV entry process in cultured insect cells (reviewed in Volkman, 1986). According to this model, BV virions enter cells via the endocytic pathway during which virions are invaginated into the cytoplasm as clathrin-coated vesicles. In the cytoplasm, the clathrin coat dissociates and the uncoated vesicles containing
25 virions fuse with cellular vesicles to form endosomes. Following the decrease of pH in the endosome, presumably by an ATP-driven proton pump, the BV envelope fuses with the endosomal membrane through the function of gp64, and release the nucleocapsid into the cytoplasm.

The entry of ODV into the midgut epithelial cells is
30 believed to occur through the fusion of the ODV envelope with the

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plasma membrane of the midgut cells (reviewed in Adams and McClintock, 1991).

2.   Nucleocapsid uncoating

After nucleocapsids are released into the cytoplasm, they
5 are transported to the nucleus for uncoating.

3.   Progeny nucleocapsid assembly

Progeny nucleocapsids are assembled within the nucleoplasm of infected cells.

4.   Envelopment of progeny viruses

10   The envelopes of ODV and BV are derived from distinct cellular sources and appear to exhibit marked tissue specificity (Adams and McClintock, 1991). ODV appears to be specialized both for interaction with polyhedrin and for infection of the epithelial cells in the insect midgut. BV is specialized for interaction with other
15 cell types and tissues in the insect. ODV is responsible for spreading infection among the natural insect hosts, whereas BV is responsible for the secondary infection within the infected host and in cultured insect cells. There are dramatic differences in the infectivity of ODV and BV both *in vivo* and *in vitro* as well as in
20 their ability to be neutralized by homologous and heterologous antisera (Volkman *et al.*, 1976; Volkman and Summers, 1977; Keddie and Volkman, 1985). Differences in infectivity correlate with the different roles of these two viral forms in the virus life cycle (Blissard and Rohrmann, 1990).

25   In the early phase, nucleocapsids destined for BV escape the nucleus, possibly by budding through both outer and inner nuclear membranes, and reach the plasma membrane in an unenveloped state. Then, nucleocapsids bud through the plasma membrane to become BV virions. Budding appears to occur at gp64-containing plasma
30 membrane regions with a "spiny coat" appearance. BV also contains a "spiny coat" structure called peplomer at one end of the virion. In the mature BV, gp64 is distributed throughout the envelope, but

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appears more concentrated in the peplomer, thus constituting the
major protein component of the peplomer.

In the late phase, ODV are enveloped from membrane
structures, microvesicles, induced within the nucleoplasm of infected
5 cells. Intranuclear morphogenesis of microvesicles begins at
approximately 12 h. p.i. The microvesicles have a unit membrane
structure and they normally appear as open-ended membranes as well as
thin tubular membranes. ODV nucleocapsids display polarity in their
association with microvesicles. In many cases, before the completion
10 of envelopment, they are seen lined with each other with the capped
end attached to the membrane. After ODV obtains its envelope, it is
further occluded into the viral occlusion which is released after
cell lysis and is very stable in the environment for many years.

Maturation of the ODV envelope within the nucleus is very
15 unique compared to herpes simplex virus (HSV) and plant
nucleohabdovirus (Jackson et al., 1987; Roizman and Sears, 1990).
Maturation of HSV results in initial envelopment of nucleocapsids in
the perinuclear space, but the progeny viruses are further
transported through the cytoplasm to the exterior of the cell
20 (Roizman and Sears, 1990). In contrast, there is no evidence that
ODV ever leaves the nucleus for ODV envelope proteins to be further
modified in the cytoplasm after their incorporation into the ODV
envelope. During HSV infection, the inner nuclear membrane is
modified in regions where nucleocapsids bud, but unit-membranes are
25 not observed in the nucleoplasm. In contrast, baculoviruses induce
extensive elaboration of unit-membranes within the nucleoplasm and
budding of nucleocapsids through unit-membranes occurs at the
intranuclear membranes.

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Summary of the Invention

In general, a novel polypeptide, designated 23AA, is identified and characterized. The 23AA polypeptide is the N-terminal 23 amino acids of ODV-E66 and includes a hydrophobic domain. This nucleotide sequence encodes for a transport polypeptide which directs the localization of fused desired protein(s) to occlusion derived virus envelopes.

For this invention, the phrase "transport polypeptide" can also be described as a "retention polypeptide," as a "target polypeptide," or as a "signal polypeptide." In this respect, "transport polypeptide" is meant to include a "retention polypeptide," a "target polypeptide" and a "signal polypeptide."

This amino acid sequence can serve as a targeting signal, as shown with β-galactosidase (β-gal), green fluorescent protein (GFP) and URF-13 protein, for incorporating many different proteins, having potentially any function, into the occlusion derived viral (ODV) envelope.

This small amino acid sequence has been fused to a number of proteins, all with differing sites of localization, e.g., β-gal (a protein that is normally cytoplasmic), green fluorescent protein (GFP; a protein that usually resides in both the cytoplasm and nucleus) and URF-13 (a protein that normally resides within the plasma membrane). The experimental data confirms that these marker proteins are targeted and reside within the ODV envelope of the recombinant viruses and intranuclear viral-induced microvesicles. Prior to this invention, there were no known methods to accomplish protein targeting to the ODV envelope and intranuclear microvesicles.

The N-terminal 23 amino acids are composed of the following amino acids:

Met-Ser-Ile-Val-Leu-Ile-Ile-Val-Ile-Val-Val-Ile-Phe-Leu-Ile-
1   5   10   15

Cys-Phe-Leu-Tyr-Leu-Ser-Asn-Ser
        20   23

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The invention includes a substantially pure DNA encoding a transport polypeptide, a strand of which DNA will hybridize at high stringency to a probe containing a sequence of at least 15 consecutive nucleotides of Fig. 1B (23AA). The protein encoded by the
5 DNA of the invention may share at least 80% sequence identity (preferably 85%, more preferably 90%, and most preferably 95%) with the amino acids listed in Fig. 1B

More preferably, the DNA includes the coding sequence of the nucleotides of Fig. 1B, or a degenerate variant of such a sequence.
10 Also included in this invention, is isolated DNA having a nucleotide sequence selected from the group consisting of: (a) the nucleotide sequence of Fig. 1B; and (b) nucleotide sequences which, through the degeneracy of the genetic code, encode the same peptide gene product as that encoded by the nucleotide sequence of Fig. 1B.

15   The probe to which the DNA of the invention hybridizes preferably consists of a sequence of at least 20 consecutive nucleotides, more preferably 40 nucleotides, and most preferably 50 nucleotides, of the coding sequence of the nucleotides listed in Fig. 1B or the complement thereof. Such a probe is useful for detecting
20 expression of 23AA transport polypeptide in a biological sample by a method including the steps of (a) contacting a biological sample with an antibody or antibody fragment made against 23AA, and (b) determining whether the antibody or antibody fragment binds to a component of the sample, such binding being an indication that the
25 sample contains 23AA transport polypeptide having a hydrophobic domain.

This invention also includes a substantially pure DNA containing a sequence of at least 15 consecutive nucleotides (preferably 20, more preferably 30, even more preferably 50, and most
30 preferably all) of the region from nucleotides 122 to 190 of the nucleotides listed in Fig. 1A.

By "high stringency" is meant DNA hybridization and wash conditions characterized by high temperature and low salt concentration, e.g., wash conditions of 65°C at a salt concentration

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of approximately 0.1 x SSC, or the functional equivalent thereof.
For example, high stringency conditions may include hybridization at
about 42°C in the presence of about 50% formamide; a first wash at
about 65°C with about 2 x SSC containing 1% SDS; followed by a second
5 wash at about 65°C with about 0.1 x SSC.

By "substantially pure DNA" is meant DNA that is not part of
a milieu in which the DNA naturally occurs, by virtue of separation
(partial or total purification) of some or all of the molecules of
that milieu, or by virtue of alteration of sequences that flank the
10 claimed DNA. The term therefore includes, for example, a recombinant
DNA which is incorporated into a vector, into an autonomously
replicating plasmid or virus, or into the genomic DNA of a prokaryote
or eukaryote; or which exists as a separate molecule (e.g., a cDNA or
a genomic or cDNA fragment produced by polymerase chain reaction
15 (PCR) or restriction endonuclease digestion) independent of other
sequences. It also includes a recombinant DNA which is part of a
hybrid gene encoding additional polypeptide sequence, e.g., a fusion
protein. Also included is a recombinant DNA which includes a portion
of the nucleotides listed in Fig. 1A which encodes an alternative
20 splice variant of 23AA.

The DNA may have at least about 70% sequence identity to the
coding sequence of the nucleotides listed in Fig. 1B, preferably at
least 75% (e.g. at least 80%); and most preferably at least 90%. The
identity between two sequences is a direct function of the number of
25 matching or identical positions. When a subunit position in both of
the two sequences is occupied by the same monomeric subunit, e.g., if
a given position is occupied by an adenine in each of two DNA
molecules, then they are identical at that position. For example, if
7 positions in a sequence 10 nucleotides in length are identical to
30 the corresponding positions in a second 10-nucleotide sequence, then
the two sequences have 70% sequence identity. The length of
comparison sequences will generally be at least 30 nucleotides, and
most preferably at least 50 nucleotides. Sequence identity is
typically measured using sequence analysis software (e.g., Sequence

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Analysis Software Package of the Genetics Computer Group, University
of Wisconsin Biotechnology Center, 1710 University Avenue, Madison,
WI 53705).

   The invention also includes a vector containing a DNA
5   encoding a polypeptide which includes the amino acid sequence of Fig.
1B, e.g., a construct in which the coding sequence is operably linked
to a suitable promoter or other regulatory sequences for expression
of the polypeptide, and a cell containing such a vector. This vector
includes a suitable promoter that is compatible to the system used
10   and directs the expression of the desired protein. The cell may be
procaryotic or eukaryotic and preferably expresses the recombinant
polypeptide encoded by the nucleotides listed in Fig. 1B.

   A "vector" is defined as a replicable nucleic acid
construct, e.g., a plasmid or viral nucleic acid. Vectors may be
15   used to amplify and/or express nucleic acid encoding 23AA protein.
An expression vector is a replicable construct in which a nucleic
acid sequence encoding a polypeptide is operably linked to suitable
control sequences (promoters) capable of effecting expression of the
polypeptide, e.g., 23AA, in a cell. The need for such control
20   sequences will vary depending upon the cell selected and the
transformation method chosen. Generally, control sequences include a
transcriptional promoter and/or enhancer, suitable mRNA ribosomal
binding sites, and sequences which control the termination of
transcription and translation. Methods which are well known to those
25   skilled in the art can be used to construct expression vectors
containing appropriate transcriptional/translational control signals.
See for example, the techniques described in Sambrook et al., 1989,
Molecular Cloning: A Laboratory Manual (2nd Edition), Cold Spring
Harbor Press, N.Y., which are incorporated by reference. A gene and
30   its transcription control sequences are defined as being "operably
linked" if the transcription control sequences effectively control
the transcription of the gene. Vectors of the invention include, but
are not limited to, baculovirus expression vectors and other viral
vectors. Preferred viral vectors of the invention are those derived

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from retroviruses, adenovirus, adeno-associated virus, SV40 virus, or
herpes viruses.

As stated above, the invention features a cell preferably
expressing the recombinant polypeptide encoded by the nucleotides
5   listed in Fig. 1B. This cell can be a prokaryotic cell, e.g., an
Escherichia coli cell, or a eukaryotic cell. Eukaryotic cells that
can be used in the invention include, but are not limited to, COS,
CHO, HeLa, TN368, Sf21, and Sf9 cells. In the case of a eukaryotic
cell, the gene may or may not be integrated into the genome of the
10 cell. Also included in the invention is an essentially homogeneous
population of prokaryotic or eukaryotic cells, each of which contains
(i.e., is transfected with) a recombinant 23AA gene. Transfection
can be transient or stable, and if desired can be carried out *in vivo*
or *ex vivo*, using the patient's own cells.

15   The invention also includes a substantially pure DNA 23AA
protein (a) containing a hydrophobic domain; and (b) localizing to
occlusion derived virus envelopes thereby directing the localization
of fused or hybrid proteins to occlusion derived virus envelopes.

Preferably, the 23AA transport polypeptide includes the
20 amino acid sequence of Fig. 1B, e.g., in the form of a 23AA fusion
protein. By "polypeptide" is meant any chain of amino acids,
regardless of length or post-translational modification (e.g.,
glycosylation or phosphorylation). The amino acid sequence of the
transport polypeptide preferably differs from the amino acid sequence
25 of Fig. 1B solely by conservative amino acid substitutions, e.g.,
substitution of one amino acid for another of the same class (e.g.,
valine for alanine, arginine for lysine, etc.) or by one or more non-
conservative substitutions, deletions, or insertions located at
positions of the amino acid sequence where the changes do not destroy
30 the function of the transport polypeptide (e.g., to direct the
localization of fused or hybrid proteins to occlusion derived virus
envelopes).

Preferably, the amino acid sequence of the hydrophobic
transport polypeptide, 23AA, is at least 80%, more preferably 85%,

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more preferably 90%, and most preferably 95% identical to the amino
acid sequence of Fig. 1B.

     By a "substantially pure transport polypeptide" is meant a
polypeptide which has been separated from at least some of those
5 components which naturally accompany it. Typically, the polypeptide
is substantially pure when it is at least 60%, by weight, free from
the proteins and other naturally-occurring organic molecules with
which it is naturally associated in vivo. Preferably, the purity of
the preparation is at least 75%, more preferably at least 90%, and
10 most preferably at least 99%, by weight. A substantially pure 23AA
polypeptide may be obtained, for example, by extraction from a
natural source (e.g., baculovirus ODV-E66); by expression of a
recombinant nucleic acid encoding an 23AA polypeptide; or by
chemically synthesizing the polypeptide. Purity can be measured by
15 any appropriate method, e.g., column chromatography such as
immunoaffinity chromatography using an antibody specific for 23AA,
polyacrylamide gel electrophoresis, or HPLC analysis. A protein or
polypeptide is substantially free of naturally associated components
when it is separated from at least some of those contaminants which
20 accompany it in its natural state. Thus, a polypeptide which is
chemically synthesized or produced in a cellular system different
from the cell from which it naturally originates will be, by
definition, substantially free from its naturally associated
components. Accordingly, substantially pure proteins or polypeptides
25 include eukaryotic proteins synthesized in *E. coli*, other
prokaryotes, or any other organism in which they do not naturally
occur.

     Also included in this invention is substantially pure DNA
that includes a sequence of at least 15 consecutive nucleotides of
30 substantially pure DNA from the region of nucleotides 122 to 190 of
Fig. 1A.

     Also, this invention includes substantially pure DNA that
includes a sequence of at least 15 consecutive nucleotides of
substantially pure DNA encoding the transport polypeptide 23AA and

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defined as nucleotides 122 to 190 of Fig. 1A. The amino acid sequence
of this transport polypeptide is:
M S I V L I I V I V V I F L I C F L Y L S N S.

The invention also includes fragments (e.g., antigenic
5 fragments) of the 23AA polypeptide. As used herein, "fragment," as
applied to a polypeptide, will ordinarily be at least 10 residues,
more typically at least 20 residues in length. Fragments of the 23AA
polypeptide can be generated by methods known to those skilled in the
art, e.g., by enzymatic digestion of naturally occurring or
10 recombinant 23AA polypeptide, by recombinant DNA techniques using an
expression vector that encodes a defined fragment of 23AA, or by
chemical synthesis. The ability of a candidate fragment to exhibit
a characteristic of 23AA (e.g., binding to an antibody specific for
23AA) can be assessed by methods described herein. Purified 23AA or
15 antigenic fragments of 23AA can be used to generate new antibodies or
to test existing antibodies (e.g., as positive controls in a
diagnostic assay) by employing standard protocols known to those
skilled in the art. In one preferred embodiment, a monoclonal
antibody is generated using the hydrophobic domain which corresponds
20 to an amino acid sequence of Fig. 1B to immunize an appropriate
laboratory animal, such as a mouse. Also included in this invention
are polyclonal antisera generated by using 23AA or a fragment of 23AA
as the immunogen in, e.g., rabbits. Standard protocols for
monoclonal and polyclonal antibody production known to those skilled
25 in this art may be employed. The monoclonal antibodies generated by
this procedure can be screened for the ability to identify
recombinant 23AA cDNA clones, and to distinguish them from known cDNA
clones.

Also included in the invention are 23AA polypeptides which
30 are encoded at least in part by portions of Fig. 1B, e.g., products
of alternative mRNA splicing or alternative protein processing
events, or in which a portion of 23AA sequence has been deleted or
altered. The fragment, or the intact 23AA polypeptide, may be

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covalently linked to another polypeptide, e.g. which acts as a label, a ligand or a means to increase antigenicity.

The invention also includes a polyclonal or monoclonal antibody which specifically binds to 23AA. Preferably, this antibody

5   specifically binds to an epitope in the hydrophobic domain of 23AA which corresponds to the sequence shown in Fig. 1B. The invention encompasses not only an intact monoclonal antibody, but also an immunologically-active antibody fragment, e.g., a Fab or (Fab)$_2$ fragment; an engineered single chain Fv molecule; or a chimeric

10   molecule, e.g., an antibody which contains the binding specificity of one antibody, e.g., of murine origin, and the remaining portions of another antibody, e.g., of human origin.

In preferred embodiments, the antibody, or a fragment thereof, may be linked to a toxin or to a detectable label, e.g. a

15   radioactive label, non-radioactive isotopic label, fluorescent label, chemiluminescent label, paramagnetic label, enzyme label, or colorimetric label.

Examples of suitable toxins include diphtheria toxin, *Pseudomonas* exotoxin A, ricin, and cholera toxin.

20   Examples of suitable enzyme labels include malate hydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, alcohol dehydrogenase, alpha-glycerol phosphate dehydrogenase, triose phosphate isomerase, peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase,

25   glucose-6-phosphate dehydrogenase, glucoamylase, acetylcholinesterase, etc.

Examples of suitable radioisotopic labels include ^{3}H, ^{125}I, ^{131}I, ^{32}P, ^{35}S, ^{14}C, etc.

Paramagnetic isotopes for purposes of *in vivo* diagnosis can

30   also be used according to the methods of this invention. There are numerous examples of elements that are useful in magnetic resonance imaging. For discussions on *in vivo* nuclear magnetic resonance imaging, see, for example, Schaefer et al., (1989) *JACC* 14, 472-480; Shreve et al., (1986) *Magn. Reson. Med.* 3, 336-340; Wolf, G. L.,

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(1984) *Physiol. Chem. Phys. Med. NMR* 16, 93-95; Wesbey et al., (1984) *Physiol. Chem. Phys. Med. NMR* 16, 145-155; Runge et al., (1984) *Invest. Radiol.* 19, 408-415.

Examples of suitable fluorescent labels include a
5 fluorescein label, an isothiocyalate label, a rhodamine label, a phycoerythrin label, a phycocyanin label, an allophycocyanin label, an ophthaldehyde label, a fluorescamine label, etc.

Examples of chemiluminescent labels include a luminal label, an isoluminal label, an aromatic acridinium ester label, an imidazole
10 label, an acridinium salt label, an oxalate ester label, a luciferin label, a luciferase label, an aequorin label, etc.

Those of ordinary skill in the art will know of other suitable labels which may be employed in accordance with the present invention. The binding of these labels to antibodies or fragments
15 thereof can be accomplished using standard techniques commonly known to those of ordinary skill in the art. Typical techniques are described by Kennedy et al., (1976) *Clin. Chim. Acta* 70, 1-31; and Schurs et al., (1977) *Clin. Chim. Acta* 81, 1-40. Coupling techniques mentioned in the latter are the glutaraldehyde method, the periodate
20 method, the dimaleimide method, the m-maleimidobenzyl-N-hydroxy-succinimide ester method. All of these methods are incorporated by reference herein.

Also within this invention, is a method of directing a desired protein to occlusion derived virus envelopes which includes
25 the steps of: (a) constructing a vector comprising a marker protein, e.g., beta-galactosidase, and the DNA of claim 2; (b) fusing a nucleotide sequence encoding a desired protein to the DNA of Step (a) to form a hybrid vector; (c) incubating the hybrid vector of Step (b) with a biological sample; and (d) determining the localization of the
30 marker protein within the biological sample, wherein localization indicates the presence of occlusion derived virus envelopes and also delivery of the hybrid vector to the envelopes.

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USES AND ADVANTAGES

As described herein, this invention, an N-terminal twenty three amino acid (23AA) hydrophobic sequence, capable of targeting desired proteins to the ODV envelope, provides a number of uses and
5 advantages. In addition to the many uses and advantages described above, this unique 23AA sequence can be used in the following ways.

For example, the 23AA sequence can be used to target desired proteins, with known properties or desired characteristics, to the ODV envelope.

10   This invention will also be useful in identifying the signals and pathways for the transport and assembly of membrane proteins into the nucleus and/or the use of specific targeting signals from ODV-E66 to locate potentially any protein, peptide, or derivatives to the nuclear envelope or other nuclear membranes.

15   Additionally, this 23AA sequence of this invention can be used to locate a protein, peptide or derivative of such (23AA-protein product) to cytoplasmic membranes, the nuclear envelope, the inner nuclear membrane or intranuclear membranes induced during viral infection or during specific stage(s) of cell cycle.

20   Examples of insect infected cells, include, but are not limited to (a) Sf9 cells, Sf21 cells or related strains, (b) TN368 cells or related strains or (c) any insect cell (or invertebrate cell) infected by a baculovirus (e.g., AcMNPV) expressing a structural or functional protein cognate containing the 23AA sequence
25 or its structural/functional equivalent.

Examples of viral infection of insects include, but are not limited to (a) *Heliothis virescens* larvae or related susceptible strains; (b) *Trichoplusia ni* larvae or related susceptible strains; or (c) any insect larvae species susceptible to infection by a
30 baculovirus.

The use of the claimed 23AA sequence for directing the location of 23AA-protein product in a desired cell would include, but not limited to, the delivery of proteins, peptides or derivatives thereof for (a) insecticidal effects - insect pest control; (b)
35 therapeutic applications (e.g., the localization of a protein,

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peptide or derivative into the nuclear envelope which will alter the
cell cycle); and (c) diagnostics (e.g., insert a marker specific for
a disease or abnormality into a cell tissue manifesting such
abnormalities).

5   **Expression of the 23AA-protein Product in Infected Cells**
           The 23AA-protein product of this invention can be expressed
    in infected cells as a single copy, multiple copies of the same
    protein or in variable combinations with other proteins (e.g., 23AA-
    protein A, 23AA-protein B, ... 23AA-protein X). The 23AA-protein
10 product of this invention can also be expressed in the infected cell
    at any time during the infection process by expression from promoter
    classes representing immediate early (0-4 hpi), delayed early (4-6
    hpi), late (6-24 hpi) or very late (24 hpi-5 days or more).
           Also, the 23AA-protein product can be directed to locate to
15 the desired locations in vertebrate, plant or other cell types during
    viral infection of that organism or cell. This can be done by
    placing the 23AA-protein product under the expression of any viral
    gene of any viral promoter class engineered for expression during
    infection of vertebrate, plant or microbial cells. This could
20 therefor introduce the 23AA-protein product into the cell during the
    infection cycle to deliver the desired protein into cytoplasmic or
    nuclear membranes.

    Expression of the 23AA-protein Product in Uninfected Cells
           Additionally, any 23AA-protein product can be expressed in
25 uninfected cells by using gene promoters that function in cells in
    the absence of or during viral infection. For example, the desired
    23AA-protein product construct can be expressed in uninfected insect
    cells by the AcMNPV-IE1 or other immediate early gene promoters
    regulated by the cell DNA-dependent RNA polymerase; or, expressed
30 from a baculovirus (or other) gene promoter transactivated by an

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immediate early gene; or, a host cell gene promoter which is
constitutively expressed, cell cycle or developmentally regulated.

Examples of transfected, but uninfected cells, include, but
are not limited to, (a) Sf9 cells or related strains (Sf21, etc.);
5   (b) TN368 cells or related strains; and (c) any insect or
invertebrate cells, vertebrate cells, plant cells, or microbial cells
capable of transfection.

Also, examples of transformed, but uninfected cells,
include, but are not limited to, (a) Sf9 cells or related strains
10 (Sf21, etc.); (b) TN368 cells or related strains; and (c) any insect
cells, invertebrate cells, vertebrate cells, plant, or microbial
cells capable of transformation.

Additionally, the 23AA-protein product taught in this
invention can be delivered to insect cells, insects, vertebrates,
15 plant and microbial cells or organisms for targeting to the
endoplasmic reticulum, nuclear envelope and intranuclear membranes by
a variety of artificially developed or genetically engineered
strategies. For example:

(1)   The use of artificial membranes (liposomes) to deliver 23AA-
20 protein product to the cell surface which could be then targeted to
ER and nuclear envelope by the cellular endocytic and vesicular
retrograde transport pathways

(2)   A retrovirus engineered to target a specific cell or tissue
type (e.g., retroviruses), could carry in the viral envelope a
25 specific 23AA-protein product or, have incorporated in the viral
genome sequence, information specific for expression, translation and
targeting of the derived 23AA-protein product during or after
germline transformation of the cell.

(3)   The nucleotide sequence for a derived 23AA-protein product
30 could be incorporated into a protein or polyprotein which would be
processed at a specific time in the cellular or viral protein
processing pathway for incorporation into cytoplasmic membranes,
nuclear envelope, or induced nuclear membranes.

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Also, there are many possibilities for deriving or obtaining a 23AA sequence or its structural and functional cognate for any of the uses as described above. For example, the 23AA sequence may be derived from, for example, but not limited to: (1) the ODV-E66 gene

5   sequence; (2) a synthetic DNA sequence with codon usage for a particular cell species or organism optimized for the 23AA. This would include the use of codons not preferred by the baculovirus nucleotide sequence which would translate the same amino acid because of degeneracy in the code; (3) a synthetic 23AA peptide or a

10 structural and functional cognate of amino acids made synthetically or derived in part or whole from another naturally occurring protein sequence; (4) the ODV-E66 genes or proteins of other baculoviruses; and (5) the ODV-E25 protein from any baculovirus with a similar hydrophobic sequence.

15   **DEFINITIONS**

For the purpose of this invention, certain phrases and words are defined or used as follows:

Purified polyhedra and viral occlusions are used interchangeably. Polyhedra purified from insect larva are stable for

20 years.

Polyhedra derived virus or "PDV" and occlusion derived virus or "ODV" are used interchangeably.

By "BEVS" is meant baculovirus expression vector system.

By "h p.i." or "hr p.i." is meant hours post infection.

25   By "β-gal" is meant beta-galactosidase and these terms are used interchangeably.

                          *   *   *

Other features and advantages of the invention will be apparent from the following detailed description, and from the

30 claims.

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Brief Description of the Drawings

Fig. 1A is a diagram showing the nucleotide and amino acid sequences of ODV-E66.

Fig. 1B is a diagram showing the nucleotide and amino acid
5   sequences of the N-terminal 23 amino acid of ODV-E66 (nucleotide numbers 122 to 190).

Fig. 2 is an outline summarizing the different constructs, e.g., Construct #1 - Construct #12, used in this invention.

Fig. 3A, 3B and 3C are photographs showing immunogold
10 labeling of purified ODV and wild type AcMNPV infected Sf9 cells at 48 hr p.i.

Figs. 4A, 4B, and 4C are photographs showing immunogold labeling of Sf9 cells infected with pVL1393-E66F at 48 hr p.i.

Figs. 5A and 5B are photographs showing immunogold labeling
15 of Spodoptera frugiperda (Sf9) and Trichoplusia ni (TN368) cells infected with 23β-gal virus at 48 hr p.i.

Figs. 6A-6I are photographs showing localization of 23GFP in pVL1393-23GFP infected Sf9 cells.

Figs. 7A and 7B are photographs showing immunogold labeling
20 of Sf9 cells infected with pAcUW21-23URF13 virus at 48 hr p.i.

Figs. 8A-8H are photographs showing transient transfection of uninfected Sf9 cells with pIE1HR4-23GFP.

Figs. 9A and 9B are photographs showing fluorescence microscopy of mammalian COS-1 cells transiently transfected with
25 pCMV-23GFP.

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Figs. 10A and 10B are diagrams showing an overview of
directed delivery to target cell and subsequent gene delivery.

Fig. 11 is a diagram showing levels of protein production
utilizing BEVS technology and comparing amounts of foreign protein
5   produced in tissue culture and larva.

Fig. 12 is genomic map of *Autographa californica* NPV showing
a construct containing dual promoters and which is occlusion positive
and contain both NT-URF13 and WT t-URF13.

Fig. 13 is a chart showing potential applications for the
10 23AA sequence as described in the Examples.

Brief Description of the Tables

Table 1 outlines how the 23AA hydrophobic sequence of this
invention functions differently from the other reported non-
baculovirus inner nuclear membrane proteins.

15   Table 2 outlines known hydrophobic sequences identified in
baculovirus ODV envelope proteins.

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Detailed Description

AcMNPV nuclear maturation serves as a unique system to study membrane biogenesis in the nucleus and current research indicates the involvement of the membranes of the nuclear envelope in this process.

5   Study of this process may yield insights into the biogenesis of the membranes of the nuclear envelope and, in a broader sense, help to understand how eukaryotic cells assemble their elaborate network of intracellular membranes. Understanding the molecular basis of the synthesis, trafficking and assembly of ODV envelope proteins into the

10 nucleus is of utmost importance. Marker proteins specific to the ODV envelope have been identified, one of which is ODV-E66. Thus, these experiments were designed:

1)   To identify the gene encoding ODV-E66 and analyze its transcriptional and translational expression;

15 2)   To study its function in ODV nuclear maturation;

3)   To investigate the nuclear source of intranuclear viral-induced membranes

4)   To study ODV envelope protein synthesis, trafficking and assembly into intranuclear viral-induced membranes with the

20   aim to identify specific protein targeting signals and viral or cellular transport factors;

5)   To reduce to practice the identified targeting signals in a variety of insecticidal, animal, and human health applications.

25 **ODV-E66 is an Integral Membrane Protein of the ODV Envelope**

A baculovirus late gene encoding a 66 kDa structural protein (ODV-E66) of AcMNPV has been mapped, cloned, sequenced, and its expression in infected cells studied. ODV-E66 is an integral membrane protein of the ODV envelope. In the infected cell nucleus,

30 ODV-E66 is present in the intranuclear viral-induced microvesicles and ODV envelope, providing evidence that the microvesicles function as an immediate precursor in the assembly of the ODV envelope.

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In _infected_ cells, ODV-E66 localizes to the ODV envelope, intranuclear microvesicles, membranes of the nuclear envelope, and cytoplasmic membranes in close juxtaposition to the nuclear envelope. A mutant virus in which the ODV-E66 gene was replaced with a fusion
5   gene encoding the N-terminal 125 amino acids of ODV-E66 and _E. coli_ β-galactosidase (β-gal) showed that the 125-amino acid N-terminal domain targeted the β-gal fusion protein to the intranuclear microvesicles and ODV envelope. This invention specifically deals with a unique N-terminal hydrophobic sequence of 23 amino acids which
10 is derived from the N-terminal 125 amino acids of ODV-E66. In infected insect cells this 23-amino acid sequence targets β-gal, green fluorescent protein (GFP), and URF-13 to the membranes of the nuclear envelope which are further transported into the intranuclear microvesicles and ODV envelope.
15   In _uninfected_ insect and mammalian (COS-1) cells, this sequence targets GFP to the membranes of the nuclear envelope. This amino acid sequence also inserted β-gal into microsomal membranes in the rabbit reticulocyte _in vitro_ translation system. This is the first report of a unique sequence which can specifically target
20 foreign proteins to the nuclear envelope, intranuclear microvesicles, and ODV envelope and has important applications in insecticide, human, and animal health.

        Unique Features of the 23AA Sequence of this Invention Not Found in Other Membrane Proteins
25   Listed below is a summary of known hydrophobic protein sequences of inner nuclear membrane proteins reported in the literature (see Table 1 below). Except for HSV-1 gB (protein #2 below), all of the proteins listed in Table 1 below are non-viral proteins. Also, all of these proteins are located in either the
30 nuclear envelope, the nuclear pore, and/or cytoplasmic membrane. Of interest, except for the rat gp210 protein, all of the other listed proteins localize to the inner nuclear membrane (INM).
        The Table 1 below outlines how the 23AA hydrophobic sequence of this invention functions differently from the other reported non-

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baculovirus inner nuclear membrane proteins. Specifically, in infected cells, the 23AA sequence of this invention targets proteins to intranuclear viral-induced membranes, in addition to protein targeting to nuclear envelope and cytoplasmic membranes.

5   Also, in uninfected Sf9 and COS-1 cells, the targeting of proteins to membranes of the nuclear envelope (NE) correlates with cell cycle-regulated movement of integral membrane proteins.

The following abbreviations are used in Table 1: **LBR** - Lamin B receptor; **INM** - Inner nuclear membrane; **TM** - Transmembrane
10 domain; **NE** - Nuclear envelope; **HSV** - Herpes simplex virus; **gB** - Glycoprotein B; **ER** - Endoplasmic reticulum; **LAP** - Lamina associated protein; and **NPC** - Nuclear pore complex.

Table 1: Inner Nuclear Membrane Proteins

Protein	Location	Summary
Chicken LBR (COS-7 cells) Smith and Blobel, 1993 and Soullam and Worman, 1993	INM (not detected on ER, Golgi, and cell surface)	1. Chicken LBR has 8 potential transmembrane segments. The first segment is from #206-226. TM: FGTFMLMFFLPATVLYLVLMC 2. Region from #205-246, including the first TM, when fused to the C-terminus of β-gal, is sufficient to target fusion protein to INM. 3. The nucleoplasmic domain (191 aa) of LBR, when fused to the TM and C-terminal domain of chicken heptic lectin (a type II protein with internal signal sequence-the TM), targets the fusion protein to INM. The nucleoplasmic domain of LBR, when expressed alone, is in the nucleoplasm.
HSV-1 gB (COS-1 cells) Gilbert et al., 1994	NE-INM (also ONM, ER, Golgi, and cell surface)	1. gB is targeted to INM in the immature N-glycoform. 2. TM of gB is form aa.#727-795 and consists of three hydrophobic segments. 3. Segment 3 is sufficient to target VSV G protein to nuclear envelope when it replaces the TM of G protein in the fusion. Note that the fusion protein still has the signal peptide from G protein and also localizes to ER, Golgi, and cell surface.
Rat LAP2 (Hela cells) Furukawa et al., 1995	INM (not detected on ER, Golgi, and cell surface)	1. LAP2 has 452 amino acid; type II protein with 409 aa., nucleoplasmic domain; TM is between #410-433. TM: VPMWIKMLLFALVAGFLFLYQAM 2. TM is likely to function as membrane anchor and is not required or sufficient for INM targeting. Domain between #298-370 is essential, but not sufficient for Triton X-100 stable INM targeting. Domain #298-370 possibly functions to bind lamina and thus retain protein in INM.

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Table 1: Inner Nuclear Membrane Proteins

	Membrane domain of NPC	
Rat gp210 (Mouse Balb/c 3T3 cells) Wozniak and Blobel, 1992		1. gp210 is an integral membrane N-glycoprotein. 2. 1886 aa in total; TM-#1806-1818; 58 aa. C-terminal domain. TM: SYQVMFFTFFALLAGTAVTILAY 3. TM domain of gp210 is sufficient to target CD8-TM fusion to NPC (CD8 is a plasma membrane protein with an internal signal/stop transfer sequence; the TM and C-terminal domain of CD8 were deleted in the fusion). 4. The 58 aa. C-terminal domain of gp210 is a weak, but sufficient NPC targeting signal when fused after the TM domain of CD8 (the CD8 C-terminal domain was replaced).
Otefin (Drosophilia) Harel *et al.*, 1989 Paden *et al.*, 1990	INM	1. Targeting signal is unknown.
Turkey p18 (erythrocytes) Simos *et al.*, 1996	INM	1. p18 is a component of a complex consisting of LBR, lamins, LBR kinase, p34, and p18. 2. Targeting sequence is unknown.
Rat LAPlC Senior and Gerace, 1988 Martin *et al.*, 1995	INM	1. 506 aa in total; TM is between #311-333; type II protein with N-terminal nucleoplasmic domain. 2. Targeting signal is unknown.

Table 2:
Summary of Hydrophobic Sequences in
Baculovirus ODV Envelope Proteins
(Structural and Functional Cognates of 23AA of ODV-E66)

Protein	Location	Sequence
Autographa californica multinucleocapsid nuclear polyhedrosis virus (AcMNPV)		
ODV-E66 Hong et al., 1994, 1996	N-terminus (23 aa)	MSIVLIIVIVIFLICFLYLSNS
ODV-E25 Ayres et al., 1994 Hong et al., 1996	N-terminus (24 aa)	MWGIVLLIVLLIFYLYWTNALNF
ODV-E56 Theilmann et al., 1996	Internal (19 aa)	IILLIGAVLFLGLIFYFIY
ODV-E18 Braunagel et al., 1996b	Internal (19 aa)	MFLTILAVVVIIALIIIFV
Orgyia pseudotasugata multinucleocapsid nuclear polyhedrosis virus (OpMNPV)		
ODV-E25 Russell and Rohrmann, 1993	N-terminus (19 aa)	MWGALILLILVFLFYLWY
ODV-E56 Theilmann et al., 1996	Internal (19 aa)	LIWLIGAVLFLALVVYLIY

Table 2, Continued

Bombyx mori multinucleocapsid nuclear polyhedrosis virus (_BmMNPV_)

ODV-E66   N-terminus (21 aa)   MSTVLIIVVVVIFLICFWCLL
Maeda et al., 1996 *
(GenBank Access #L33180)

ODV-E25   N-terminus (18 aa)   MWKIVLLIVLLVLIYLYW
Maeda et al., 1996 *
(GenBank Access #L33180)

ODV-E56   Internal (19 aa)   IIMMIGAVLFLGLILYFIY
Theilmann et al., 1996

ODV-E18   Internal (19 aa)   MFLTILAVVIIALIIMFV
Braunagel et al., 1996b

Helicoverpa zea singlenucleocapsid nuclear polyhedrosis virus (_HzSNPV_)

ODV-E56   Internal (19 aa)   IIVVLGIVLLIIFIGYIVI
Theilmann et al., 1996

ODV-E18   Internal (19 aa)   MLMTILIALVIIILLIMLF
Braunagel et al., 1996b

Choristoneura fumiferana multinucleocapsid nuclear polyhedrosis virus (_CfMNPV_)

ODV-E56   Internal (19 aa)   LIWLIGAVLFLGLIIYLIY
Theilmann et al., 1996

Table 2, Continued

Cydia pomonella granulosis virus (CpGV)

ODV-E56   Internal (19 aa)   ILLVIGGILLLTFIGFVIF
Theilmann et al., 1996
* This is not published and the sequence was obtained from GenBank accession #L33180.

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EXAMPLES

The following examples illustrate selected modes for carrying our the claimed invention and are not to be construed as limiting the specification and claims in any way. These examples are provided so

5 as to enable one of ordinary skill in the art to make and use the invention. These examples are not intended to limit the scope of what the inventors regard as the invention. Efforts have been made to ensure accuracy with respect to numbers used to characterize the conditions; however, some experimental errors and deviations may be

10 present.

EXAMPLE 1
MATERIALS AND METHODS USED FOR THIS INVENTION

Insect Cells and Viruses

Wild type AcMNPV (E2 strain), β-gal mutant viruses, and viruses

15 overexpressing 23GFP, 23URF-13 or ODV-E66 were used to infect *Spodoptera frugiperda* (Sf9) cells at a multiplicity of infection (moi.) of 20 (Summers and Smith, 1987). One of the β-gal mutant virus, 23β-gal (Fig. 2, Construct #4) was also used to infect *Trichoplusia ni* (TN368) cells (moi. = 20). Time zero was defined as

20 the time when cells were inoculated with the virus. The virus inoculum was removed after 1 hr absorption.

Virus Purification, Fractionation, ODV-E66 Band Isolation, and N-Terminal Amino Acid Sequencing

To purify the virus, media and cells were collected 5 days

25 postinfection and centrifuged at 9000 rpm in a Beckman JA14 rotor. BV was purified from the supernatant essentially as described by Summers and Smith (1987). The supernatant was centrifuged at 100,000 x *g*, 4°C (Beckman Type 35, 30,000 rpm) to pellet the virus. Virus derived from 500 ml of cell supernatant was resuspended in 1 ml

30 0.1x TE (TE = 10 mM Tris, 1.0 mM EDTA, pH 7.4) and overlaid onto an 11 ml 25-56% sucrose gradient in 0.1x TE. Gradients were centrifuged at 100,000 x *g* for 90 min at 4°C (Beckman SW41, 24,000 rpm). The

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virus band was removed, diluted 1:4 in 0.1x TE, and re-pelleted by centrifugation at 100,000 x *g* for 30 min at 4°C (SW41, 24,000 rpm). The virus was resuspended in 0.1x TE (approximately 0.5 ml per liter of cell supernatant) and stored at 4°C.

5   Viral occlusions were isolated from infected cells by the method of Whitt and Manning (1987) with some modifications. Cells harvested above (approximately 5×10^9 cells) were resuspended in 15 ml of 0.2% Triton X-100 and lysed by sonication on ice (6 X 30 sec, output 5, duty cycle 50%; Model W-375 sonicator; Heat Systems Ultrasonics,

10   Inc.). The sonicates were brought to 80 ml with 0.2% Triton X-100 and the lysate was laid over four 10 ml 30% sucrose (w/v)/0.2% Triton X-100 pads. Viral occlusions were pelleted by centrifugation at 9000 rpm for 20 min in Beckman JA-21 rotor. Each pellet of partially purified viral occlusions was resuspended in 3-4 ml of 0.2% Triton X-

15   100, laid onto a 35 ml 35-60% (wt/wt) sucrose gradient made in water, and centrifuged at 100,000 x*g* for 30 min at 4°C (Beckman SW28, 24,000 rpm). The viral occlusion band was removed and washed twice by diluting in H_2O and low speed centrifugation. The viral occlusions were resuspended in a small volume of H_2O and stored at 4°C. Protein

20   concentration was determined by absorbance at 550 nm (extinction coefficient = 0.28; Summers and Smith, 1987).

ODV was purified from viral occlusions by the method of Tweeten et al., (1980) with some modifications. ODV was liberated from viral occlusions (40 mg/ml) by incubating at 37°C for 2 hr in diluted

25   alkaline solution (0.1 M $NaCO_2$, 0.5 M NaCl, pH 10.9). Approximately 1 ml of alkali-treated viral occlusions was laid onto an 11 ml 25-56% (w/v) sucrose/10 mM Tris (pH 7.5) gradient and centrifuged at 50,000 x *g* for 30 min at 4°C (SW41, 17,500 rpm). Multiple ODV bands were removed, washed by diluting in 10 mM Tris (pH 8.5), pelleted, and

30   finally resuspended with 10 mM Tris (pH 8.5).

Purified viruses were fractionated into envelope and nucleocapsid preparations using modifications of the protocol of Whitt and Manning (1987). In a 250 µl reaction, 200 µg of BV or ODV were incubated in 1% NP40, 10 mM Tris, pH 8.5 at room temperature for 30 min with

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gentle agitation. The solution was then laid onto a 4 ml 30-70%
(wt/vol) glycerol/10 mM Tris (pH 8.5) gradient and centrifuged at
150,000 x *g* for 60 min at 4°C (Beckman SW60, 34,000 rpm). The
envelope fraction was then recovered from the top of the gradient,

5   while the nucleocapsids formed a band approximately 2/3 into the
gradient. Both fractions were dialyzed against 10 mM Tris (pH 7.4)
overnight at 4°C. All fractions were concentrated using an Amicon
Centricon-3 microconcentrator.

For N-terminal amino acid sequencing, purified viral envelope and
10   nucleocapsid fractions were denatured in disruption buffer (2% SDS,
1% β-mercaptoethanol, 25 mM Tris, 7% glycerol, and 0.1% bromophenol
blue, pH 6.8) and proteins were separated on a 3% stacking/12.5%
separating gel that was pre-run for 30 min with 200 mM thioglycolic
acid added to the upper buffer. The separated proteins were
15   electrophoretically transferred to the PVDF membrane (Pro-Blott;
Applied Biosystems). Following transfer, the membrane was stained
(45% methanol, 5% acetic acid, 0.1% Coomassie blue R250), destained
(45% methanol, 5% acetic acid), and washed with water. Two protein
bands were excised and N-terminal sequenced. The sequence for a 66
20   kDa band unique to the ODV envelope was determined to be MSIVLIIVI
(Biotechnology Instrumentation Facility, Univ. of Calif., Riverside,
CA). A degenerate oligonucleotide was predicted from these amino
acids and used to screen AcMNPV genomic DNA digested with *PstI* by
standard Southern-blot techniques (Sambrook *et al.*, 1989).

25   **Plasmid cloning and DNA sequencing**

Plasmid cloning was described by Sambrook *et al.*, (1989). Double-
stranded DNA sequencing was done according to Sequenase 2.0 protocols
(USB) using α-^{35}S-dATP (DuPont NEN Research Products).
Oligonucleotides were synthesized on a Model 391 PCR-MATE DNA
30   Synthesizer (Applied Biosystems Inc.). DNA was sequenced on both
strands and each nucleotide was sequenced an average of six times.
The nucleotide and predicted amino acid sequences were analyzed using
the programs of genetics computer group (Devereux *et al.*, 1984;

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UWGCG, version 8.0; Madison, WI). The completed gene sequence has
been deposited with the GenBank Data Library under Accession No.
M96360.

Heterologous protein expression and antibody production

5   The region from amino acid 24 to 705 of the ODV-E66 ORF was
amplified by polymerase chain reaction (PCR; 1 min at 94°C, 1 min at
37°C, and 3 min at 72°C with 5 sec extension after each cycle, 25
cycles) using two primers (forward: 5'-
AAAAAAGCTTGTTAACAATAAAAATGATGCC-3' and reverse: 5'-AAAAGGATCCTTACACAA

10   TTTCAAA-3') and Taq polymerase (Promega).

       The resultant PCR fragment was extracted with phenol/ chloroform,
precipitated with ethanol, digested with *HindIII* and *BamHI*, separated
on a 1% agarose gel, purified (GeneClean II, BIOL101, Inc.), and
ligated into the *HindIII* and *BamHI* sites of pUC18 to give rise to

15   PCR-pUC18. The FLAG epitope (amino acid sequence: DYKDDDDK;VWR
Scientific) was cloned in frame at the N-terminus of the amplified
region (amino acids 24-705) to allow affinity purification of the
fusion protein when necessary. A linker containing *HindIII* sites on
both ends (underlined below) was cloned into the *HindIII* site of the

20   PCR-pUC18 clone. The forward sequence of the linker is shown below:
5'-AAGCTTCATATGGACTACAAAGACGACGACGACAAGCTT-3'. Immediately following
the *HindIII* site on the left, there is an *NdeI* site (italic)
containing an ATG in frame with the ODV-E66 ORF. DNA sequencing
confirmed that the final construct encodes the following N-terminal

25   amino acid sequence: MDYKDDDDKLVN (amino acid 24 of ODV-E66 ORF is
underlined). The N-terminal eight amino acids (italic) encode the
FLAG epitope. Finally, the FLAG-E66 ORF was cloned into the NdeI and
BamHI sites of pET-11a (Novagen, Inc.) and expressed. The
recombinant protein was found in the inclusion fraction of *E. coli*.

30   Bacterial inclusion fraction was prepared as described by the
manufacturer (Novagen).

       Three New Zealand White female rabbits were injected
intramuscularly with approximately 500 μg of the inclusion fraction

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in Freund's complete adjuvant (Pierce). The rabbits were then given
two additional injections in Freund's incomplete (Pierce) at monthly
intervals. One week after the final injection, sera were collected
by exsanguination of the rabbits. To eliminate the background
5 reaction, the polyclonal antiserum was preadsorbed with uninfected
Sf9 cells. Uninfected Sf9 cells were pelleted, resuspended in 1 X
BLOTTO (1% non-fat dry milk, 50 mM Tris, 150 mM NaCl, 0.05% Tween-20,
0.02% NaN₃, pH 7.4), and sonicated until all cells were lysed. ODV-
E66 antiserum or preimmune serum was added (1:500) and allowed to
10 bind overnight at 4°C. The antibody was then clarified by
centrifugation for 20 min at 15,000 rpm in a Beckman JA-21 rotor.
Preadsorbed polyclonal antiserum and preimmune serum were stored at -
20°C until use.

To prepare a better antibody which does not require preabsorption
15 to be monospecific for ODV-E66, induced crude *E. coli* lysate was
separated on a 10% SDS-PAGE gel. Gel strips were cut from both sides
of the gel, stained with Coomassie blue (Sambrook *et al.*, 1989),
aligned with an unstained part of the same gel, and the band
containing the expressed protein was excised. Then, the unstained
20 gel slice was forced through a gauge 22 needle before letting protein
diffuse out of gel by shaking in 50 ml TBS (50 mM Tris, 150 mM NaCl,
pH 7.4) overnight at 4°C. Diffused protein was concentrated in a
Centriprep-30 (Amicon). The protein concentration was determined by
Bradford assay (Bradford, 1976). Two New Zealand White female
25 rabbits were injected subcutaneously four times with a total of 220
ug of protein per rabbit in RIBI adjuvant (RIBI ImmunoChem Research)
as described by Harlow and Lane (1988). Nine days after the final
injection, sera were collected by exsanguination. Preimmune sera
were collected before the first injection. Western-blot analyses
30 showed that the antiserum was monospecific for ODV-E66.

Immunoelectron microscopy (IEM)

To prepare samples for IEM, infected Sf9 or TN368 cells,
transfected Sf9 cells, and freshly purified viruses were pelleted and

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sequentially fixed: 1% paraformaldehyde, 0.5% glutaraldehyde, 0.05 M
sodium cacodylate, pH 7.1, 10 min at 4°C; 2% paraformaldehyde, 2.5%
glutaraldehyde, 0.05 M sodium cacodylate, pH 7.1, 30 min at 4°C (all
EM chemicals except gold-conjugates were purchased from Electron
5   Microscopy Science). Fixed pellets were washed (0.05 M sodium
cacodylate, pH 7.1; 3 X 10 min at 4°C), postfixed (1% osmium
tetraoxide, 0.05 M sodium cacodylate, pH 7.1; 30 min at room
temperature), and washed again. After fixation, samples were
dehydrated and infiltrated following a protocol described by
10   Vandenbosch (1991). Briefly, the pellets were dehydrated at 4°C with
30% ethanol for 30 min and then at -20°C with the following graded
ethanol series: 50%; 70%; 90%; 100% (30 min/step). Samples were
infiltrated with 50%, 75%, 100% LR White/ethanol series -20°C (1 hr
per step). Samples were then infiltrated with 100% LR White at -20°C
15   overnight, followed by another 8 hr infiltration. The resin was
polymerized in gelatin capsules heated to 50°C for approximately 48
hr.

Ultrathin sections were cut on a Reichert-Jung ultracut E
microtome and collected on nickel grids (Electron Microscopy
20   Sciences), etched (0.56 M sodium meta-periodate, 1 hr; Sigma), washed
(H_2O, 2 min), incubated in 0.1 N HCl (10 min), and washed (H_2O, 4 X 2
min). The sections were blocked with TTBS-BSA (1% BSA, 50 mM Tris-
HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.4) for 15 min, reacted with
primary antibody (1:500 E66 preabsorbed antiserum, 1:1250 β-gal mouse
25   ascitic fluid, or 1:1500 GFP antiserum; 16 hr at 4°C), and washed (50
mM Tris, 150 mM NaCl, pH 7.4; 4 X 2 min). The bound primary antibody
was detected using anti-rabbit IgG 15 nm or 30 nm gold conjugate or
anti-mouse IgG 30 nm conjugate (Amersham). The sections were stained
with uranyl acetate (Bozzola and Russell, 1992) and lead citrate
30   (Venable and Coggeshall, 1965), and visualized with a Zeiss 10C
transmission electron microscope (Electron Microscopy Center,
Department of Biology, Texas A&M University, College Station, TX).

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Deletion and site-directed mutagenesis

To construct the 125β-gal plasmid, the KpnI-KpnI fragment (28.1-28.2 m.u., Fig. 1a, Hong et al., 1994) within the PstI-F region (23.5-30.1 m.u.; cloned into pUC19) was replaced with a KpnI linker,

5   5'-GGTACCAA<u>GGATCC</u>TTGGTACC-3' (BamHI site is underlined). A 3.1 kb BamHI fragment containing the *lacZ* gene from plasmid pAc360-β gal (Luckow and Summers, 1989) was inserted into the BamHI site so that the region encoding N-terminal 125 amino acids of ODV-E66 was fused in frame with the *lacZ* gene.

10   To introduce partial deletions within the N-terminal 125 amino acids of 125β-gal, the EcoRI-K region (25.0-29.2 m.u., Fig. 1a, Hong et al., 1994) containing wild type *ODV-E66* was cloned into the EcoRI site of pUC19 as the template for mutagenesis using transformer site-directed mutagenesis kit (ClonTech; See Construct #2, Fig. 2, "125β-

15   gal"). Both deletion and site-directed mutagenesis were done as instructed by the manufacturer. All mutants were confirmed by DNA sequencing (Sequenase 2.0; USB).

To obtain the construct Δ2-23β-gal (See Construct #3, Fig. 2), amino acids #2-23 were deleted using the deletion oligonucleotide 5'-

20   GCAACATTCGACATGAGCAATAATAAAAATGATGCC-3' and the selection oligonucleotide 5'-CTCTAGAGGAACCCCGGGAACCGAGCTCG-3'. After the introduction of deletion, the KpnI-KpnI fragment (28.1-28.2 m.u.) was replaced with the 3.1 kb KpnI fragment containing the *LacZ* gene from 125β-gal. Construct #3 is the same as construct #2 except that the

25   amino acids #2-23 (22 amino acids total) are deleted. The fusion protein Δ2-23β-gal is cytoplasmic.

To obtain the construct 23β-gal (See Construct #4, Fig. 2), nucleotide sequence encoding amino acids #24-25 were mutated to a KpnI site using the selection oligonucleotide 5'-CTCTAGAGGAACCCCGGG

30   AACCGAGCTCG-3' and the site-mutation oligonucleotide 5'-CCTATCAAATAGC <u>GGTACC</u>AAAAATG ATGCCAAT-3' (KpnI site is underlined). The region from the new KpnI site at amino acids #24-25 to the KpnI site at 28.2 m.u. was replaced with the 3.1 kb KpnI fragment containing the *LacZ* gene from 125β-gal.

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Cloning strategy for overexpression of 23GFP

The N-terminal 23 amino acids of ODV-E66 (MSIVLIIVIVVIFLICFLYLSNS) was amplified by PCR (30 sec at 94°C, 60 sec at 42°C, 90 sec at 72°C, 25 cycles) using the following oligonucleotides: 5'-
5   TTTTTTAAGCTTATGTCTATCGTATTG-3' (HindIII site is underlined) and 5'-TTTTTTGGATCCTTGCTA TTTGATAGGTA-3' (BamHI site is underlined). The PCR product was extracted with chloroform, precipitated with ethanol, digested with HindIII and BamHI, gel purified, and cloned into the HindIII and BamHI sites of pUC19. Amplified region was verified by
10 DNA sequencing. The BamHI/EcoRI fragment from pGFP vector (ClonTech) which contains the complete GFP open reading frame (ORF) was cloned into the BamHI/EcoRI sites of the pUC19 PCR clone so that N-terminal 23 amino acids of ODV-E66 were fused in frame with GFP (pUC19-23GFP).

The clone pVL1393-23GFP (See Construct #6, Fig. 2) was generated
15 to overexpress 23GFP in insect cells. The HindIII site in pUC19-23GFP was filled by Klenow and the HindIII-EcoRI fragment was cloned into the SmaI/EcoRI sites of transfer vector pVL1393 (Webb and Summers, 1990). In this construct, expression of 23GFP was controlled by the polyhedrin promoter. Infected cells have an
20 occlusion minus phenotype.

The 23GFP ORF was then cloned into the transfer vector pAcUW21 (PharMingen). The region from the BamHI site in the multiple cloning region of pVL1393-23GFP to the EcoRI site at the 3'-end was cloned into the BglII/EcoRI sites of pAcUW21 (ClonTech). In this construct
25 (pAcUW21-23GFP; See Construct #5, Fig. 2) expression of 23GFP was under the control of the p10 promoter. Infected cells have an occlusion positive phenotype.

A recombinant virus containing GFP under the control of the polyhedrin promoter was provided by Dr. Christian Oker-Blom (VTT
30 Biotechnology, Finland; Laukkanen et al., 1996; Oker-Blom et al., 1996) and was used as a control for immunofluorescence and immunogold labeling.

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Cloning strategy for overexpression of ODV-E66

To express ODV-E66 at high levels under the polyhedrin promoter and in the polyhedrin locus, *ODV-E66* was amplified by PCR (1 min at 94°C, 1 min at 37°C, and 3 min at 72°C with 5 sec extension after each
5 cycle, 25 cycles) using the following oligonucleotides: 5'-TTTTTGGATCCACCATGTCTATCGTATTG-3' (BamHI site underlined) and 5'-TTTTTCTGCAGTTACTTGTCGTCGTCGTCTTTGTAGTCCACAATTTCAAAAAT-3' (PstI site underlined). The second oligonucleotide contains the "FLAG" epitope (italic; amino acid sequence: DYKDDDDK; VWR Scientific) immediately
10 upstream of the stop codon. The PCR product was cloned into the BamHI and PstI sites of pVL1393 (Webb and Summers, 1990). The resultant construct (pVL1393-E66F; See Construct #7, Fig. 2) was sequenced to verify that PCR did not introduce errors in the nucleotide sequence.

15 ### Cloning strategy for overexpression of 23URF13

To overexpress a fusion gene encoding N-terminal 23 amino acids fused in frame with URF13 gene (Dewey et al., 1986) under the control of p10 promoter in the pAcUW21 transfer vector, recombinant PCR was used to amplify and combine the nucleotide sequence encoding
20 23 amino acids of ODV-E66 with the URF13 gene. N-terminal 23 amino acids of ODV-E66 was amplified by PCR (60 sec at 94°C, 30 sec at 42°C, 60 sec at 72°C, 25 cycles) using the following oligonucleotides: 5'-TTTTCGGGATCCATGTCTATCGTATTG-3' (BamHI underlined) and 5'-TAAGAAAGTAGTGATGCTATTTGATAGGTA-3'. URF13 gene was amplified by PCR
25 (60 sec at 94°C, 30 sec at 42°C, 60 sec at 72°C, 25 cycles) using the Turf-2b plasmid as template (Dewey et al., 1986) and the following oligonucleotides: 5'-TACCTATCAAATAGCATCACTACTTTCTTA-3' and 5'-AAAAAGGAATTCTCACGGTACTTGTAC-3' (EcoRI site underlined). After the first round of PCR for 23 amino acids and the URF13 gene, 1 ul of PCR
30 product from each PCR reaction was used as the templates for a second round of PCR (60 sec at 94°C, 30 sec at 42°C, 60 sec at 72°C, 25 cycles) using two of the oligonucleotides used in the first PCR: 5'-TTTTCGGGATCCATGTCTATCGTATTG-3' (BamHI underlined) and 5'-

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AAAAAG<u>GAATTC</u>TCACGGTACTTGTAC-3' (EcoRI site underlined). Product from
the second PCR was extracted with chloroform, precipitated with
ethanol, digested with BamHI and EcoRI, and cloned into the
BglII/EcoRI sites of pAcUW21 vector. Recombinant virus made using
5 this construct (pAcUW21-23URF13; See Construct #8, Fig. 2) produces
viral occlusions in infected cells.

Construction of Recombinant Viruses

Recombinant viruses 125β-gal, Δ2-23β-gal, and 23β-gal, were
constructed using the corresponding transfer plasmid and AcMNPV viral
10 DNA (Summers and Smith, 1987). pVL1393-23GFP, pAcUW21-23GFP,
pVL1393-E66F, and pAcUW21-23URF13 were constructed using the
corresponding plasmid constructs and Bsu36I-digested BakPak6 viral
DNA (ClonTech). The locus of recombination was verified by Southern
analyses and an appropriate probe. Protein expression was confirmed
15 by Western-blot analyses of infected cell extracts using appropriate
antibody: β-gal recombinant viruses (mouse anti-β-gal polyclonal
antibody at 1:1250; Sigma); 23GFP recombinant viruses (rabbit anti-
GFP polyclonal antibody at 1:1500; ClonTech). Southern and Western-
blot analyses were done as described by Sambrook et al., (1989).
20   Fig. 12 is genomic map of *Autographa californica* NPV showing a
construct containing dual promoters and which is occlusion positive
and contain both 23URF13 and wild type t-URF13 (WT t-URF13).
Multiple genes can be inserted at both the polyhedrin and p10 loci.
In this manner, recombinant viruses can be constructed that are
25 occlusion positive and contain multiple genes. Genomic map modified
and reprinted from Ayres, et al., Virology, 202:586-605 (1994).
Construction of recombinants containing multiple genes is routine for
the inventors using the techniques described by Belyaev and Roy,
Nucleic Acid Research, 21:1219-1223 (1993) and Summers and Smith,
30 Texas Agricultural Experiment Station, Bulletin No. 1555 (1987).

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GFP Autofluorescence

Autofluorescence of GFP in cells expressing GFP or 23GFP was observed on a Zeiss Axiophot microscope (Electron Microscopy Center, Texas A&M University) equipped with a filter set for fluorescein
5 isothiocyanate (FITC). Cells were photographed using phase contrast, fluorescence, or double exposure of phase contrast and fluorescence. Exposure time for cells at different time points postinfection was maintained constant and was set for proper exposure of the 72 hr p.i. time point for comparison of fluorescence intensity.

10 **Transient Expression of 23GFP in Uninfected Sf9 Cells**

To transiently express 23GFP under the control of the IE1 promoter, the HindIII/EcoRI fragment from 23GFP-pUC19 was filled with Klenow at both ends and cloned into the PmeI site of pIE1HR4 (Jarvis et al., 1996) so that the filled HindIII site was close to the BamHI
15 site in pIE1HR4. This construct was named pIE1HR4-23GFP (See Construct #9, Fig. 2).

Transfection of plasmid DNA into uninfected Sf9 cells was done using the CellFECTIN lipofection reagent as instructed by the manufacturer (Gibco BRL). For each transfection, 2 ug of plasmid DNA
20 and 9 ul of CellFECTIN reagent were used. Cells were recovered at 24 or 48 hr post-transfection for fluorescence observation or EM fixation. The pattern of protein localization was indistinguishable between 24 and 48 hr post-transfection.

Transient expression of 23GFP in mammalian (COS-1) cells
25 The pUC19-23GFP construct (see Cloning Strategy for Overexpression of 23GFP) was digested with EcoRI, filled with Klenow (Sambrook et al., 1989), Klenow enzyme inactivated by heating at 75°C for 15 min, and digested with HindIII. Then, the resulting HindIII/EcoRI fragment containing 23GFP was cloned into the HindIII/HpaI sites of
30 pCMV Blue vector (PharMingen). In this construct (pCMV-23GFP; See Construct #10, Fig. 2) expression of 23GFP is under the control of

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the major immediate early promoter of cytomegalovirus which is active
in the COS-1 cells.

COS-1 cells (Gluzman, 1981) were maintained at 37°C in Dulbecco's
modified Eagle's medium (Gibco BRL) supplemented with 10% fetal calf
5   serum. Transfection of plasmid DNA into COS-1 cells was done in DMEM
medium without serum and antibiotics using the CellFECTIN lipofection
reagent as instructed by the manufacturer (Gibco BRL). For each
transfection, 2 ug of plasmid DNA and 15 ul of CellFECTIN reagent
were used.   After transfection, cells were incubated at 37°C for 24
10 hr for 23GFP to be expressed, then cells were transferred to 30°C and
incubated for another 48 hr for GFP autofluorescence to develop
(Ogawa *et al.*, 1995).

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EXAMPLE 2
IDENTIFICATION, SEQUENCE, AND ANALYSIS OF ODV-E66
AND THE N-TERMINAL 23 AMINO ACID HYDROPHOBIC DOMAIN

This Examples teaches the identification, sequence determination
5   and analyses of ODV-E66 and the N-terminal 23 amino acid hydrophobic
    domain.

        Baculovirus Expression of AcMNPV ODV-E66 and Overexpression of
        ODV-E66 Under the Control of the Polyhedrin Gene Promoter in the
        Polyhedrin Locus

10   To identify proteins unique to the ODV envelope, ODV and BV were
    purified and fractionated into envelope and nucleocapsid fractions.
    A comparison of the structural proteins of ODV, BV, and envelopes
    revealed a protein of 66 kDa unique to the ODV envelope (Braunagel
    and Summers, 1994, incorporated herein by reference). N-terminal
15 amino acid sequencing of this protein determined the sequence of: M S
    I V L I I V I . The gene was mapped to the EcoRI-PstI region (25.0-
    30.1 m.u., Fig. 1 of Hong et al., 1994, incorporated herein) of the
    AcMNPV genome and the nucleotide sequence (2115 nucleotides of open
    reading frame) of the gene encoding ODV-E66 predicts a protein of 704
20 amino acids (Fig. 1).

        Fig. 1A is a diagram representing the nucleotide and amino acid
    sequences of the gene encoding ODV-E66. Nucleotide sequences
    upstream and downstream of ODV-E66 open reading frame are also
    included. The amino acid sequence of ODV-E66 is shown in single
25 capital letters. The numbers to the left indicate positions of
    nucleotide and amino acid. The N-terminal 23 amino acid hydrophobic
    sequence is boxed thereby including nucleotide numbers 122 through
    190 (sixty-nine (69) nucleotides). Restriction endonuclease sites
    (DraI, KpnI, and EcoRI) are underlined. Arrows above the nucleotide
30 sequences (TTAAG and ATAAG) indicate the transcription initiation
    sites.

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The nucleotide sequences and amino acid sequences for the 23AA sequence of this invention are shown below (see Fig. 1B):

```
ATG   TCT   ATC   GTA   TTG   ATT   ATT   GTC
M     S     I     V     L     I     I     V


5  ATA   GTT   GTA   ATA   TTT   TTA   ATA   TGT
   I     V     V     I     F     L     I     C


TTT   TTG   TAC   CTA   TCA   AAT   AGC
F     L     Y     L     S     N     S
```

The 23 AA sequence has the following amino acid sequence:
10 M S I V L I I V I V V I F L I C F L Y L   S N S.

Studies of the gene encoding ODV-E66 demonstrated that it is a baculovirus late gene with transcription initiating from conserved TAAG motifs (Fig. 1A, arrows; For complete description of genetic analysis including Northern-blot, primer extension, 5' and 3' S1
15 nuclease analysis see Hong et al., 1994).

Fig. 2 is an outline summarizing the different constructs used in this invention and includes the construct name, gene promoter and locus, occlusion production, and protein location in cells. Numbers above the structures indicate locations of amino acids.
20   In Construct #1, ODV-E66, the ODV-E66 expression is controlled by its native promoter in the native locus in wild type virus and infected cells produce viral occlusions.

Fig. 3A, 3B and 3C are photographs showing immunogold labeling of purified ODV and wild type AcMNPV infected Sf9 cells at 48 hr p.i.
25 Rabbit ODV-E66 antiserum and anti-rabbit IgG 15 nm gold-conjugate were used. Fig. 3A: Labeling of the viral envelope of purified ODV (arrowhead). Fig. 3B: Labeling of the intranuclear viral-induced microvesicles. Note the microvesicles are surrounded by fibrillar structures and viral occlusions. Fig. 3C: Labeling of the viral

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envelope of ODV being occluded into a viral occlusion (arrowhead).
The following abbreviations are used in this figure: m,
microvesicles; f, fibrillar structure; oc, viral occlusion.

Studies of the protein ODV-E66 show that it is a structural
5   protein of the ODV envelope (Fig. 3A, arrowheads; Hong et al., 1994).
During viral assembly, ODV-E66 locates to viral induced intranuclear
microvesicles (Fig. 3B, m) and the ODV envelope that is maturing into
the occluded form (Fig. 3C, arrowheads; Hong et al., 1994).

A recombinant virus was generated that places an additional copy
10 of the gene encoding ODV-E66 into the baculovirus genome under the
control of the polyhedrin promoter (Fig. 2, Construct #7). In
construct #7, an extra copy of ODV-E66 gene replaces the polyhedrin
gene in the polyhedrin locus in Construct #7. In this construct, (a)
expression of the extra copy of ODV-E66 gene is under the control of
15 polyhedrin promoter, (b) infected cells did not produce viral
occlusions due to the absence of a functional polyhedrin gene, and
(c) the ODV-E66 gene in the native locus is intact.

Figs. 4A, 4B, and 4C are photographs showing immunogold labeling
of Sf9 cells infected with pVL1393-E66F at 48 hr p.i. Rabbit ODV-E66
20 antiserum and anti-rabbit IgG 15 nm gold-conjugate were used. Fig.
4A: Labeling in the intranuclear microvesicles and cytoplasmic
membranes close to the nuclear envelope (dual arrow). Fig. 4B:
Labeling of the cytoplasmic membranes (dual arrow). Fig. 4C:
Labeling of the outer (arrowhead) and inner (arrow) nuclear
25 membranes. The following abbreviations are used in this figure: m,
microvesicles; n, nucleus; c, cytoplasm.

Immunoelectron microscopy of the localization of ODV-E66 in this
recombinant virus shows the same protein localization as wild type
AcMNPV, however the amount of ODV-E66 is increased significantly
30 (Fig. 4; Hong et al., 1996). By increasing the amount of ODV-E66 we
could detect the same labeled structures as wild type, but
additionally can visualize cellular locations of the protein that
were indicated by wild type virus, but were observed at low levels.
These locations include: ODV envelope (Fig. 4A and 4C), microvesicles

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and other intranuclear membranes (Fig. 4A, 4C; m), membranes closely associated with the nucleus (Fig. 4A, 4B, dual arrow), the nuclear envelope, including the inner and outer nuclear membrane (4C, INM, arrow; ONM, arrowhead).

5   Overexpression of ODV-E66 by this recombinant virus confirms that ODV-E66 locates to the nuclear membrane, an observation that was indicated with wild type virus, but difficult to confirm due to decreased levels of proteins. Additionally, pVL1393-E66F generated virus confirms that when ODV-E66 (or derived fusion) is placed in the

10 baculovirus genome at an alternative locus (i.e., polyhedrin locus) and under the control of an alternate promoter (i.e., polyhedrin or p10 or other classes of viral gene promoters), it locates to the same cellular organelles and structures as it would locate in wild type baculovirus infected cells.

15   **Analysis of the N-terminal 23 Amino Acids of ODV-E66, Development of Recombinant Viruses Containing Fusion Proteins, and Localization of Fusion Proteins During Viral Infection**

Beta-Galactosidase Protein (β-gal) as a Marker

A mutant virus was constructed in which a fusion gene encoding the

20 N-terminal 125 amino acids of ODV-E66 was fused in frame with β-galactosidase (β-gal; Fig. 2; 125β-gal, Construct #2) and replaced *ODV-E66* in the native locus.

Construct #2 shows that the ODV-E66 gene, in the native locus, is replaced by a mutant gene 125β-gal (in frame fusion of N-terminal 125

25 amino acids of ODV-E66 with β-galactosidase of *E. coli*) whose expression is still controlled by the native ODV-E66 promoter.

Because β-gal is cytoplasmic when expressed in infected Sf9 and Sf21 cells (Jarvis et al., 1991; Hershberger et al., 1994), it was expected that the fusion protein would also locate to the cytoplasm.

30 However, immunofluorescence and immunogold labeling showed that the 125β-gal fusion protein localized into the nucleus and to viral induced intranuclear microvesicles and ODV envelope (Hong, 1995). The conclusion was that the N-terminal 125 amino acids of ODV-E66 must contain the requisite signal(s) necessary to direct β-gal or

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potentially other proteins into the nucleus and intranuclear membranes.

Using computer-assisted analysis, an N-terminal hydrophobic region of 23 amino acids with a general hydrophobic character similar to the
5 signal/anchor sequences in many membrane proteins (von Heijne, 1990) was identified:

               M S I V L I I V I V V I F L I C F L Y L S N S

Several additional constructs were made which included fusion mutants with or without the N-terminal hydrophobic domain (see Fig.
10 2). After cloning the gene encoding each mutant protein, a recombinant virus was produced and expression of each gene, under a variety of baculovirus promoters, was studied. These recombinant gene constructs were then inserted at various loci within the baculovirus genome (See Fig. 2).

15   When the hydrophobic 23 amino acid sequence was fused in frame with the normally cytosolic protein β-galactosidase (23β-gal; Fig. 2, Construct #4), the fusion protein was transported into the nucleus and this was initially confirmed using fluorescent confocal microscopy (Hong, 1995). In construct #4, the ODV-E66 gene, in the
20 native locus, is replaced by a mutant gene 23β-gal (in frame fusion of N-terminal 23 amino acids of ODV-E66 with β-galactosidase of *E. coli*) whose expression is still controlled by the native ODV-E66 promoter.

      Figs. 5A and 5B are photographs showing immunogold labeling
25 of *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (TN368) cells infected with 23β-gal virus at 48 hr p.i. For these figures, mouse β-gal antiserum and anti-mouse IgG 30 nm gold-conjugate were used. Fig. 5A: 23β-gal fusion protein was localized to the intranuclear microvesicles (arrowhead) and ODV envelope (arrow) in infected Sf9
30 cells. Fig. 5B: 23β-gal fusion protein was localized to the intranuclear microvesicles (arrowhead) and ODV envelope (arrow) in infected TN368 cells. The following abbreviations are used in this figure: m, microvesicles; n, nucleus; c, cytoplasm.

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Immunoelectron microscopy of 23β-gal infected Sf9 cells showed
that the N-terminal 23 amino acids of ODV-E66 were sufficient to
direct the fusion protein 23β-gal to the viral induced intranuclear
microvesicles and ODV envelope (Fig. 5A). These results are not
5   restricted to Sf9 cells, and these results were confirmed using
another insect cell line, *Trichoplusia ni* (TN368; Fig. 5B).

Green Fluorescent Protein (GFP) as a Marker

Green fluorescent protein (GFP) is a small protein that normally
resides throughout the cells, including the cytoplasm and nucleus and
10 is not normally membrane associated. It is known that if wild type
GFP is expressed in baculovirus under the control of the polyhedrin
promoter that it also uniformly distributes throughout the cell (Hong
et al., 1996). GFP was chosen as an additional marker protein for
several reasons:
15   (a) Because it normally is not excluded from the nucleus, the
23GFP fusion should also indicate specific controls or in the
presence of specific localizations factors, for both the cytoplasmic
and nuclear compartments; and
        (b) The autofluorescence of GFP should allow one to visually
20 track and locate the fusion protein movement after viral infection.
        Thus, GFP should provide another example and an overview of
specific localization and transport with a high degree of sensitivity
and specificity for detection.
        Two recombinant viruses were generated which contained the N-
25 terminal 23 amino acids of ODV-E66 fused in frame with GFP (23GFP;
Fig. 2, Constructs #5-#6). In construct #5, a fusion gene of N-
terminal 23 amino acids with green fluorescent protein (23GFP) was
placed under the control of p10 promoter and inserted into the
polyhedrin locus as an extra copy of transcriptional unit. The
30 polyhedrin and p10 genes in their native loci are still intact, thus
infected cells still produced viral occlusions. In construct #6, the
23GFP fusion gene replaced the polyhedrin gene in the polyhedrin
locus. Expression of 23GFP was under the control of polyhedrin

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promoter. Infected cells did not produce viral occlusions due to the
absence of a functional polyhedrin gene.

Cells infected by pAcUW21-23GFP (p10 promoter) or pVL1393-23GFP
(polyhedrin promoter) were occlusion-positive or occlusion-negative,
5   respectively. Cells infected by pAcUW21-23GFP produced normal
amounts of viral occlusions and occluded virus as determined by
immunogold labeling (Hong et al., 1996). The pattern of label of
23GFP fusion protein location was essentially the same for the
occlusion positive and occlusion negative mutants. Because of the
10 additional complexity of occlusion formation within the nucleus, the
data using the occlusion-negative mutant has been chosen for the
representative observation of GFP localization (Fig. 6).

Figs. 6A-6I are photographs showing localization of 23GFP in
pVL1393-23GFP infected Sf9 cells. Fig. 6A: 48 h p.i., GFP
15 autofluorescence. Fig. 6B: 48 h p.i., GFP autofluorescence and phase
contrast. Arrows in A and B point to the fluorescence at the
periphery of the nucleus. Fig. 6C: 72 h p.i., GFP autofluorescence.
Fig. 6D: 72 h p.i., GFP autofluorescence and phase contrast.
Arrowheads in C and D point to the fluorescence in the interior of
20 the nucleus. Figs. 6E, 6F, 6G, 6H, 6I: Immunogold labeling of Sf9
cells infected with pVL1393-23GFP at 48 hr p.i. using rabbit GFP
antiserum and anti-rabbit IgG 30 nm gold-conjugate. Fig. 6E: Note
the labeling of cytoplasmic membranes at the periphery of the nucleus
and foci of intranuclear microvesicles. Fig. 6F: Labeling of
25 cytoplasmic membranes (dual arrow), outer (arrow) and inner
(arrowhead) nuclear membranes. Fig. 6G: Labeling of large
cytoplasmic vesicles (open-arrow). Fig. 6H: Labeling of outer
(arrow) and inner (arrowhead) nuclear membranes. Fig. 6I: Labeling
of the ODV envelope (arrow). The following abbreviations are used in
30 these figures: c, cytoplasm; n, nucleus; v, virogenic stroma; m,
microvesicles.

Immunoelectron microscopy showed that 23GFP located to the same
cellular membranes as the 125β-gal and 23β-gal fusion proteins and
wild type ODV-E66 (Fig. 6). 23GFP initially locates to regions

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closely associated with the nuclear envelope (Fig. 6A-6B; arrow) and as infection progresses, 23GFP moves into the nucleoplasm as discrete foci of microvesicles (Fig. 6C-6D; arrowhead). Immunogold analysis shows that the 23GFP fusion protein locates to the same cellular
5   membranes as wild type ODV-E66. These membranes include: Cytoplasmic membranes juxtaposed to the periphery of the nucleus (Fig. 6E, 6F; dual arrows; 6G, open arrows); Inner and Outer nuclear membranes of the nuclear envelope (Fig. 6F-6H; INM, arrowheads, ONM, arrows); Intranuclear foci of microvesicles (Fig. 6E, m); and, ODV envelope
10 (Fig. 6I; arrows) and other intranuclear membranes.

The data demonstrated that proteins normally cytosolic (β-gal) or freely dispersed between the nucleus and cytoplasm (GFP) are specifically trafficked into defined membranes when fused to the 23 hydrophobic amino acid sequence of ODV-E66.

15 Plasma Membrane Protein URF13 as a Marker

To confirm that the 23 amino acid sequence was also capable of directing a protein that normally locates to the plasma membrane, the plasma membrane protein URF13 was chosen for additional study. The N-terminal 23 amino acid sequence was fused to wild type URF13
20 (23URF13; Fig. 2, Construct #8) and the recombinant virus generated. In construct #8, a fusion gene of N-terminal 23 amino acids with URF13 gene was placed under the control of p10 promoter and inserted into the polyhedrin locus as an extra copy of transcriptional unit. In this construct, the polyhedrin and p10 genes in their native loci
25 are still intact, thus infected cells still produce viral occlusions.

Figs. 7A and 7B are photographs showing immunogold labeling of Sf9 cells infected with pAcUW21-23URF13 virus at 48 hr p.i. Rabbit URF13 antiserum and anti-rabbit IgG 30 nm gold-conjugate were used in these experiments. Fig. 7A: Labeling of the ODV envelope (arrow) and
30 intranuclear vesicles (open arrow). Fig. 7B: Labeling of cytoplasmic membranes (arrowhead). The following abbreviations are used in this figure: c, cytoplasm; n, nucleus.

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The data showed that 23URF3 located to the same structures as the β-gal and GFP fusion proteins, *i.e.*, the ODV envelope (Fig. 7A, arrow), intranuclear membranes (Fig. 7A, open arrow) and membranes closely associated with the nuclear envelope (Fig. 7B, arrowhead).

5   Thus, three marker proteins that normally locate to three different regions of the cell were used in this experiment

(1)   β-gal is normally cytosolic;

(2)   GFP is normally both cytosolic and nuclear; and

(3)   URF13 is usually targeted to the plasma membrane.

10 This data demonstrates that when each of these three different proteins was fused to the N-terminal 23 amino acid sequence of ODV-E66, it was targeted to different cellular locations than their wild type counterparts. Specifically these locations were: cytoplasmic membranes juxtaposed to the nuclear envelope, both the outer and

15 inner nuclear membranes of the nuclear envelope, foci of viral induced intranuclear microvesicles and the ODV viral envelope. These examples clearly showed that in baculovirus infected cells, the 23 amino acid sequence specifically located a protein to distinct membrane structures.

20 **Localization of the Fusion Proteins in Uninfected Insect Cells and Mammalian Cells --- Demonstrated Use of Alternate Promoters, and Potential Cell Cycle Regulation of Protein Location**

To determine where this 23 amino acid sequence targets a protein in underlined cells would more specifically address the role of the

25 23 amino acids and eliminate the possible role of other viral encoded proteins for the specific localization of the fusion proteins.

Thus, these experiments were performed in both uninfected insect (Sf9) cells and uninfected mammalian (COS-1) cells. To perform these experiments, promoters were chosen that are recognized by the host

30 cell RNA polymerase II. For the insect cells, the promoter from the immediate early gene IE1 of AcMNPV was used (Fig. 2, Construct #9). For the mammalian cells, the promoter of the major immediate early gene of cytomegalovirus was chosen (CMV; Fig. 2, Construct #10).

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In construct #9, the 23GFP fusion gene was placed under the
control of IE1 promoter in the pIE1HR4 vector for transient
expression in uninfected insect cells. This was different from
previous constructs #1-#8) which were recombinant viruses expressing
5   proteins in infected cells. For this construct, an IE1 promoter was
used (IE1 is an AcMNPV immediate early gene promoter that is active
in uninfected insect cells). The fusion gene construct was
transfected into Sf9 cells and transient expression was achieved
without viral infection. The results showed that in uninfected Sf9
10   cells, 23GFP protein localized to the nuclear envelope in a cell
cycle-dependent manner.

It must be emphasized that a wide variety of promoters from many
known genes could have been chosen for this study or any similar
experiment with similar results.

15   Figs. 8A-8H are photographs showing transient transfection of
uninfected Sf9 cells with pIE1HR4-23GFP. Figs 8A, 8C, 8E:
Fluorescence microscopy at 24 hr post-transfection. Figs. 8B, 8D,
8F: Matched fluorescence and phase contrast image double exposure to
fluorescence images in Figs. 8A, 8C, 8E, respectively. Figs. 8G, 8H:
20   Immunogold labeling of binucleated cells at 48 hr post-transfection
using rabbit GFP antiserum and anti-rabbit IgG 30 nm gold-conjugate.
Fig. 8G: Note small vesicles in the nucleoplasm (arrowhead) and
cytoplasmic vesicles juxtaposed to the nuclear envelope (dual arrow
in insert). Fig. 8H: Labeling of cytoplasmic vesicles (open arrow),
25   outer and inner nuclear membranes (arrow). Also note the
invagination of the inner nuclear membrane into the nucleoplasm
(arrowhead). The following abbreviations are used in this figure: c,
cytoplasm; n, nucleus; ne, nuclear envelope.

Two general patterns of fluorescence were observed in insect Sf9
30   cells that were transiently transfected with IE1-23GFP:

(a)   Some cells showed diffuse fluorescence indicating that 23GFP
was located throughout the cell (Fig. 8C, 8D, 8E, 8F); and

(b) Dividing cells, as determined by the presence of two nuclei in
the same cell, showed an enhancement of fluorescence around the

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periphery of the nucleus or in the nuclear envelope (Fig. 8A, 8B, 8C, 8D: arrows).

Immunogold labeling of cells harvested at 48 hr post transfection confirmed these observations. Many cells showed labeling throughout

5   the cell, both the cytoplasm and nucleus (data not shown); however, in binucleated cells, 23GFP located to vesiculated cytoplasmic membranes adjacent to the nuclear envelope (Fig. 8H, open arrows), and the outer and inner nuclear membranes (Fig. 8H, arrows). These results indicate that proteins containing the 23 amino acid sequence

10 are differentially regulated to the nuclear membrane during discrete stages of the cell cycle. Germline transformed Sf9 cells have been developed containing the 23GFP fusion protein, and studies using time lapse exposures to closely evaluate protein movement relative to cell cycle are being performed. In mitotic cells, observations show that

15 cytoplasmic membranes vesiculate and locate in close proximity to the nuclear envelope (Fig. 8G, ne, and insert; dual arrows) in a manner very analogous to the type of membrane localization observed in baculovirus infected cells. Thus, scientific discoveries of specific pathways of protein movement in baculovirus infection may extend

20 significantly to currently unknown pathways of protein movement for nuclear membranes and membranes within the nucleus in other cell types that may be regulated by the cell cycle.

Next, a 23GFP fusion product under the control of a promoter which is optimized for mammalian cell expression was constructed (Fig. 2;

25 #10). In construct #10, the 23GFP fusion gene was placed under the control of the major immediate early gene promoter of cytomegalovirus (CMV-IE) in the pCMV vector for transient expression in uninfected mammalian COS-1 cells. The fusion gene construct was transfected into COS-1 cells and transient expression was achieved without viral

30 infection.

In uninfected COS-1 cells, 23GFP protein localized to the nuclear envelope in a cell cycle-dependent manner. Figs. 9A and 9B are photographs showing fluorescence microscopy of mammalian COS-1 cells transiently transfected with pCMV-23GFP. Fig. 9A: Note the ring of

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fluorescence (arrowhead) in a cell with two nuclei that is likely in mitosis. Fig. 9B: The ring of fluorescence is not obvious. This cells is possibly not in the mitosis due to the presence of a single nucleus and intact nuclear envelope (arrowhead). Using the pCMV-
5 23GFP construct the results demonstrate that autofluorescence of 23GFP is enriched along the nuclear membranes of bi-nucleated (*ie.*, mitotic) cells (Fig. 9A), while in non-mitotic cells the fluorescence is diffuse (Fig. 9B). This data predicts and supports that the fusion protein containing the ODV-E66 N-terminal 23 amino acids is
10 specifically located to the membranes closely associated with the nucleus, including the inner nuclear membrane, at specific times during the cell cycle. This data also predicts that this sequence functions in a similar manner in both insect and mammalian cells.

EXAMPLE 3
15   **EXPRESSION OF 23 GREEN FLUORESCENT PROTEIN IN PLANT CELLS**

This Example presents a method for constructing a plant vector containing the N-terminal 23 hydrophobic amino acids from ODV-E66 and was designed to study the localization of protein (green fluorescent protein) as a result of the 23 amino acid sequence.

20   To express 23 green fluorescent protein (23GFP) in plant cells, the 23GFP fusion gene amplified by two rounds of PCR in order to be cloned into the plant expression vector pRTL2 (Carrington and Freed, 1990). The first round of PCR includes two PCR reactions (94°C, 30 sec; 42°C, 60 sec; 72 °C, 60 sec; 25 cycles): The first reaction used
25 the pAcUW21-23GFP construct as template and two oligonucleotides (5'-TTTTTTCCATGGCATCTATCGTATTGATT-3' and 5'-GACAAGTGTTGGCCAGGGAACAGGTAGTTTTCC-3'); the second reaction used the pAcUW21-23GFP construct as template and two oligonucleotides (5'-GGAAAACTACCTGTTCCCTGGCCAACACTTGTC-3' and 5'-
30 TTTTTTTCTAGACTATTTGTATAGTTC-3'). The second round of PCR used 1% of each of the first round of PCR reactions and two oligonucleotides (5'-TTTTTTCCATGGCATCTATCGTATTGATT-3' and 5'-TTTTTTTCTAGACTATTTGTATAGTTC-3'; NcoI and XbaI sites underlined). PCR fragment from the second PCR was extracted with chloroform,

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precipitated with ethanol, digested with NcoI and XbaI, purified from
1% agarose gel using GeneClean kit (BIOL101), and cloned into the
NcoI/XbaI sites of pRTL2 vector. This construct was named pRTL2-
23GFP (Fig 2., Construct #11). Thus, in this construct, the 23GFP
5 fusion gene was placed under the control of the 35S promoter in the
pRTL2 vector for expression in plant cells. 35S promoter is active
in plant cells without viral infection.

       23GFP autofluorescence in plant cells can be observed using a FITC
filter in a Zeiss Axiophot microscope (Zeiss). Experiments are in
10 progress to determine the location of 23GFP in plant cells.

                              EXAMPLE 4
                  EXPRESSION OF A FUSION GENE OF N-TERMINAL
                  24 AMINO ACID HYDROPHOBIC SEQUENCE OF ODV-E25
                        WITH GFP IN INSECT SF9 CELLS
15   ODV-E25 is another ODV envelope protein of baculovirus OpMNPV
(Russell and Rohrmann, 1993),and AcMNPV (Ayres et al., 1994), is
related to ODV E66, and it is the N-terminal 24 amino acids of AcMNPV
ODV-E25 that has been used in this Example. This Example presents a
method for constructing a vector containing the 24 N-terminal amino
20 acids from ODV-E25 and forming a fusion product. This construct
would be used to identify the presence of a functional cognate in
these 24 amino acids.

       AcMNPV ODV-E25 was localized to the microvesicles (T. Hong, S. C.
Braunagel, and M. D. Summers, unpublished observation) and also
25 contains an uncleaved N-terminal 24 amino acid hydrophobic sequence
(Hong et al., 1996). To determine if this 24 amino acid sequence
functions similarly to the 23 amino acid sequence of ODV-E66, a
fusion construct (24GFP) was made in which the 24 amino acid sequence
of ODV-E25 was fused in frame with GFP. The nucleotide sequence
30 encoding the N-terminal 24 amino acids of ODV-E25 was amplified by
PCR (94°C, 30 sec; 42°C, 60 sec; 72°C, 60 sec; 25 cycles) using
purified AcMNPV viral DNA (Summers and Smith, 1987) as template and
two oligonucleotides: 5'-TTTTTTCTGCAGATGTGGGGAATCGTG-3', PstI site
underlined; 5'-TTTTTTGGATCCTTGAAATTTAATGCATT-3', BamHI site

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underlined. The PCR product was extracted with chloroform, precipitated in ethanol, digested with PstI and BamHI, purified from 1% agarose gel using GeneClean kit (BIOL101), and cloned into PstI/BamHI sites of pUC19 vector. The PstI/BamHI PCR insert in pUC19
5   and the BamHI/EcoRI fragment from the pGFP vector (ClonTech) containing the GFP gene were cloned into the PstI/EcoRI sites of pVL1392 vector (Webb and Summers, 1990). This construct was named pVL1392-24GFP (See Fig. 2, Construct #12). In this construct, the N-terminal 24 amino acid hydrophobic sequence from ODV-E25 was fused in
10 frame with GFP. Recombinant virus expressing 24GFP is being made using the construct pVL1392-24GFP and Bsu36I digested BakPak6 viral DNA (ClonTech.). In this virus, the 24GFP fusion gene replaces the polyhedrin gene in the polyhedrin locus. Infected cells will not produce viral occlusions due to the absence of a functional
15 polyhedrin gene.

EXAMPLE 5
STUDIES ON THE BIOCHEMISTRY AND MOLECULAR PROCESS
OF TRANSPORT OF PROTEINS CONTAINING THE
N-TERMINAL HYDROPHOBIC DOMAIN OF ODV-E66

20   Additional studies are being performed to address the molecular basis and regulation of transport of proteins containing the N-terminal amino acid hydrophobic domain of ODV-E66. These studies demonstrate the effect on both baculovirus infected insect cells and in uninfected cells including insect, vertebrate and plant cell
25 lines. Clonal isolates of Sf9 cells that have undergone germline transformation and have a copy of the IE1-23GFP gene placed within its genome have been developed.

        The results of these studies have also been observed in vertebrate and plant cell lines which have also undergone germline
30 transformation. Additionally, wild type ODV-E66 and fusion proteins are being utilized to identify other protein factors or protein processing that may be involved in some function to assist, target, or transport proteins to membranes of the nuclear envelope.

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    These studies may suggest normal insect cells contain cellular
cognates of several of the baculovirus ODV envelope proteins, and
that these cognates may prove to be important in protein movement in
uninfected cells. Sequencing of clones for two potential cellular
5 cognates is being performed. Screening for accessory proteins that
may play a role in protein transport of ODV-E66 and several other ODV
envelope proteins is also being performed. Screening methods being
utilized in this laboratory include: yeast two-hybrid methodology,
covalent linkages of protein complexes, anti-idiotype identification
10 of binding proteins, and identification of protein complexes using
antibody isolation techniques.

                            EXAMPLE 6
              IDENTIFICATION OF OTHER HYDROPHOBIC SEQUENCES
               FOUND IN BACULOVIRUS ODV ENVELOPE PROTEINS ---
15   STRUCTURAL AND FUNCTIONAL COGNATES OF 23AA OF ODV E66

    This Example is designed to identify potential cognates from other
baculovirus genes with known hydrophobic sequences. The additional,
known, hydrophobic sequences, found in baculovirus ODV envelope
proteins, will be characterized as either structural or functional
20 cognates of 23AA of ODV-E66. It is not known if the potential
hydrophobic cognates possess the same innate ability to locate a
foreign protein, or derivative in the same way, as ODV-E66 or the
23AA sequence of this invention.

    Listed in Table 2 below are hydrophobic sequences identified in
25 baculovirus ODV envelope proteins. In order to identify structural
or functional cognates of the 23AA of ODV E66, constructs are being
made for each of these hydrophobic sequences with protocols and goes
to those disclosed above. These protocols are very similar to those
designed to produce the constructs shown in Fig. 2. The analyses as
30 shown in Example 2 are being performed to determine if a transport,
retention or signal sequence, similar to the 23AA of this invention,
is contained within the tested hydrophobic ODV domains.

    The following abbreviations are used in Table 2 below:

ACMNPV: Autographa californica multinucleocapsid nuclear
35   polyhedrosis virus

OPMNPV: *Orgyia pseudotasugata* multinucleocapsid nuclear polyhedrosis
           virus

BmMNPV: *Bombyx mori* multinucleocapsid nuclear polyhedrosis virus

HzSNPV: *Helicoverpa zea* singlenucleocapsid nuclear polyhedrosis
5   virus

CfMNPV: *Choristoneura fumiferana* multinucleocapsid nuclear
           polyhedrosis virus and

CpGV: *Cydia pomonella* granulosis virus

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EXAMPLE 7
10   **APPLICATION OF NEWLY IDENTIFIED 23 AMINO ACID**
        TARGETING SEQUENCE --- INSECTICIDES/INSECTICIDAL TOXINS

</div>

Current approaches to genetically engineer baculoviruses to serve
as insecticide delivery agent(s) utilize a recombinant baculovirus
for delivery of a gene encoding toxic agents to insects (Martens,
15 1994). For successful results, the gene of interest must be
expressed, secreted from infected cells or modified by the infected
cell, and become active in the insect hemolymph or cells. These
approaches have resulted in the insects dying faster than when
infected with wild type baculovirus. Initial indications suggest that
20 these recombinant viruses cause sufficiently rapid cessation of
feeding and will be sufficiently cost-effective to make these viral
constructs commercially viable insecticidal agents in the field.

a.   Insecticidal Toxins in General

Baculovirus ODV fuses with the insect gut cell to deliver their
25 genetic components into the infected cell. The use of the 23 amino
acid hydrophobic sequence of this invention, or structural or
functional cognate thereof, which targets proteins to the ODV
envelope, provides an effective way to directly deliver a toxin to
the cellular membranes of the insect gut (See Fig. 10A for an
30 outline). Fig. 10 is a diagram showing an overview of directed
delivery to target cell and subsequent gene delivery. Fig. 10A is a
diagram showing targeting and delivery to target cell via membrane
fusion with the purified microvesicles or ODV envelope (I-V). If the

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delivered protein has toxic activity after being incorporated into
the cell membrane, then this protein may have an insecticidal
application (VI). Fig. 10B is an extension of the delivery shown in
Fig. 10A to include gene delivery and expression. This use is either
5   insecticidal or therapeutic depending upon the function of the
protein encoded by the recombinant gene. Thus, immediately upon
infection, the direct delivery results in compromised cellular
function and integrity, and cessation of eating. In addition, the
direct delivery of toxin(s) to the insect gut cell membranes can be
10 combined with the classical gene delivery approach (See Fig. 10B).
The example described below is an example of one such toxin delivered
as described above. Other toxins, or agents that compromise cell
health, ionic pore regulators, etc. can be utilized for insecticidal
purposes using the 23 AA sequence of this invention and the protein
15 or gene delivery approach as described in this example.

   b.   Producing Insecticidal Toxin URF13 Protein and Direct
         Delivery to the Insect Midgut
      Maize exhibiting the Texas male-sterile cytoplasm (cms-T) trait
was widely used in commercial hybrid maize production before, in
20 1970, severe disease outbreaks to fungal pathogens forced the
industry to curtail its use. The URF13 protein (13 kDa) encoded by
the mitochondrial gene T-urf13, is responsible for cms-T trait and
has received considerable scientific investigation because of the
impact on commercial maize production (Dewey et al., 1986).
25 Unexpectedly, researchers observed that baculoviruses expressing
URF13 were lethal to Trichoplusia ni larva (T. ni.; cabbage looper;
Korth and Levings, 1993). Additionally, when URF13 is expressed in
baculovirus and is transported to the plasma membrane of the infected
insect cells, it confers T-toxin or methomyl (fungicide) sensitivity
30 to the cell.
      The ODV form of baculoviruses is the primary infectious agent in
the insect. The ODV envelope fuses with the plasma membrane of the
gut cells, thus immediately upon infection, viral envelope proteins
are incorporated into the gut cell plasma membrane. URF13 lethality

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in insects is independent of other factors and apparently only
requires delivery to the plasma membrane for toxic activity.

    Thus, in view of this background and discussion, the inventors
propose direct delivery of URF13 to the plasma membrane of the insect
5 gut by constructing a recombinant virus that contains URF13 in its
ODV envelope (e.g., 23URF13). This direct delivery will cause damage
to the insect gut cell, resulting in compromised insect health and a
cessation of eating (Fig. 10A). Since URF13, when located in the
plasma membrane of baculovirus infected cells, confers sensitivity to
10 methomyl, adding trace amounts of the fungicide methomyl to the
feeding formulation will increase the toxicity of the delivered
protein, e.g, URF13.   One advantage of this approach, is that it
can be combined with the more traditional approaches to deliver
gene(s) that encode additional copies of the same, or additional
15 toxins. Thus, after the insect function is compromised, via direct
delivery of a toxin to the gut, the insect will immediately be
exposed to additional toxin activity, thus decreasing its ability to
recover (Fig. 10B) and increasing the speed and efficacy of the
insecticidal effects. To increase URF13 insecticidal activity,
20 another mutant virus containing the 23URF13 and an additional copy of
the wild type URF13 under the control of the early baculovirus
promoter (IE1) may also be employed. Moreover, as will be
appreciated by those skilled in this area, additional copies of
either URF13 or other toxins, e.g., the scorpion toxin AaHIT, can
25 also be used in the delivery system of this invention, although
additional toxin delivery and reinforcement may be required.

    The time course of delivery and concentration of any of these
genes can be regulated by choice of promoter - both temporal
expression, e.g., immediate early, delayed early, late or very late,
30 and strength and activity levels of promote, as will also be
understood by those skilled in this art.

EXAMPLE 8
APPLICATION OF NEWLY IDENTIFIED 23 AMINO ACID
TARGETING SEQUENCE --- PROTEIN MEDIATED TARGETING AND
VIRUS-LIKE PARTICLES THERAPEUTIC USE

5   Baculovirus infection results in a large number of small,
       intranuclear membrane vesicles. These have been named
       "microvesicles". These microvesicles serve as one source of the ODV
       envelope and it has been shown that the 23 amino acid sequence
       locates proteins to these structures at a high efficiency (Hong et
10   al., 1994, Braunagel et al., 1996a, Braunagel et al., 1996b; Hong et
       al., 1996). Techniques are currently being tested to purify these
       microvesicles. The inventors have identified at least one protein
       that results in a greatly enhanced production of intranuclear
       microvesicles, if expressed at high levels in an infected cell
15   (Braunagel and Summers, unpublished observations). Thus, purification
       of large quantities of microvesicles from infected cells in vitro,
       appears possible. For the purposes presented here, purified
       microvesicles and "virus-like" particles are used interchangeably.
       Thus, by inserting proteins(s) that are of medical value into these
20   vesicles, virus-like particles useful themselves as a therapeutic are
       created.

       a.   Virus-Like Particle Delivery of Protein to Gut Tissue
              Baculovirus ODV fuses with insect gut cells (Kawanishi et al.,
       1972). There is no evidence that ODV randomly fuses with other
25   membrane systems. Of interest, ODV is virtually non-infectious when
       delivered into the bloodstream or is used as an infectious agent in
       vitro (Volkman and Summers, 1977). The inventors have evidence that
       the baculovirus envelope contains at least one protein homolog in its
       viral envelope that resembles a protein of human rotavirus, a virus
30   which infects humans through the gut (Braunagel et al, 1992; and
       unpublished observations). It was therefore predicted that ODV would
       fuse with mammalian gut membranes. The development of recombinant
       virus carrying defined fusion proteins, e.g., 23GFP, in the ODV
       envelope now provides the tools to confirm this prediction in vitro.

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Specific therapeutic or regulatory proteins, and derivatives thereof, may now be incorporated directly into the microvesicles and directly delivered to the microvillar membranes of the gut tissue upon ingestion. Thus, generating 23AA-fusion products of integral

5 membrane proteins selectivity incorporates the fusion proteins into the microvesicles. Upon ingestion, these proteins become transiently incorporated in gut microvillar membranes. Proteins and/or receptors that enhance or replace essential components for nutrient and/or drug absorption, are examples of this type of delivery.

10   Thus, it now appears feasible that suitable foodstuff or drug formulations can include microvesicles or artificially prepared structural or functional cognates containing the relevant accessory protein for proper absorption and/or delivery. This provides an effective direct delivery system to the intestinal microvillar

15 membranes in a mammalian system.

   b.   Virus-Like Particle Delivery Into the Bloodstreams
   A number of approaches have been designed to expose certain antigens, and/or receptors or anti-receptors to infectious agents in the bloodstream. One well characterized example is delivery of CD4.

20 CD4 is a receptor that resides on a subset of T cells and is a target for the anti-receptor gp120 of Human Immunodeficiency Virus (HIV). The recognition of CD4, by gp120, allows viral entry and subsequent viral infection (Maddon et al., 1986). If CD4 is presented to the HIV virus on another surface than T cells, it acts as a "negative

25 sink" or scavenger of the virus, thus decreasing the amount of virus that successfully infects the target cells. This strategy has been studied and shows potential as an AIDS therapeutic (Nicolau et al., 1990; Arvinte et al., 1989 and Webb et al., 1989).
   While the concept of this type of delivery system is

30 straightforward, the methods of incorporating integral membrane proteins, i.e., receptors, with a defined secondary structure and orientation into artificial membrane systems (ie. liposomes) or resealed ghosts can be very difficult. Usually only a small

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proportion of the total incorporated protein is exposed and
conformationally correct. Thus, only a small percent of protein is
functional. The advantage of using baculovirus derived microvesicles
is that the orientation of the fusion protein can be designed and
5   tested to determine functional authenticity. Once the construct is
tested and confirmed to be biologically active, all copies of the
protein should be inserted into the microvesicles in the same
conformation. Additionally, multiple proteins or protein complexes
can be inserted into the microvesicles. Use of different promoters
10 of various strengths and temporal activities, allows for the
regulation of the amount and timing of gene expression and protein
production (Belyaev and Roy, 1993). Thus, this approach provides
significant control and flexibility not only of conformation of
protein insertion, but also with a way to engineer active protein
15 complexes in membrane vesicles or virus-like particles for
therapeutic use. This approach is feasible in both animal and human
hosts.

Many different proteins can be genetically engineered to be
inserted into intranuclear microvesicles. Since ODV is not the
20 infectious form of baculovirus in cell culture and is not required
for passage *in vitro*, then, presumably, any number of proteins could
be incorporated into the ODV envelope for this purpose. Since the
production of infectious ODV is not required, some non-essential ODV
proteins could be deleted from the viral genome to generate
25 additional space in the ODV envelope for recombinant, fusion
proteins. For example, it is already known that ODV-E66 and ODV-E56
are non-essential for viral propagation *in vitro* (Hong, 1995;
Braunagel *et al.*, 1996a).

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EXAMPLE 9
APPLICATION OF NEWLY IDENTIFIED 23 AMINO ACID
TARGETING SEQUENCE --- PROTEIN MEDIATED TARGETING
AND GENE OR DRUG DELIVERY

5   **a. General Approach**

Using the observation that any protein could be targeted to the
ODV envelope, and that baculovirus can be used to reconstruct
membrane protein complexes, including specific fusion proteins, it
becomes possible to insert a receptor, etc. into the ODV envelope
10   that is specific for cell type recognition and fuses only with the
target cell. A virus that contains such engineered protein(s) on its
viral envelope could be used in several ways: (1) Direct delivery to
the target cells via specific recognition and fusion, thus direct
delivery of the protein of interest to the target cell membrane; and
15   (2) Use of specific protein complexes to direct and fuse ODV envelope
to the target cell with subsequent gene delivery using any promoter
recognized by the host cell. In this manner, the baculovirus becomes
a specific vector to target protein (ie. receptor and/or drug) or
gene delivery in both animal and human hosts. (See Fig. 10 for
20   overview).

**b.   Delivery of Protein(s) to Cellular Plasma Membranes Using
        ODV as Agent**

This example (i.e., using modified ODV to deliver proteins to the
membranes of the target cell) is similar to the example using
25   purified virus-like particles to deliver proteins, but has distinct
advantages. In an occlusion positive baculovirus, mature ODV is
incorporated into viral occlusions. Once ODV is embedded within the
viral occlusion, it is very stable. This form of the virus remains
viable for an extended period of time, several years under outdoor
30   environmental conditions, and considerably longer if stored protected
from UV light. Viral occlusions are easily purified from infected
cell lysates. The occlusion can be dissolved using an alkaline
treatment to release ODV.

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Since refrigeration and drug storage is a major problem in many
areas of the world, this invention can be used to transport
therapeutic agent(s) in the stable form of viral occlusions. At the
site of delivery, a premeasured vial of occlusions is mixed with an
5 aliquoted alkaline solution, allowed to incubate at room temperature
for a specific period of time, thereby releasing the ODV, then
neutralized with the appropriate solvent system and administered to
the patient.

     c.   Gene and/or Drug Delivery to Target Cell Using ODV as Agent
10   This example utilizes the ability to direct ODV to the target cell
by incorporating receptors, fusion proteins etc. into the ODV
envelope. Upon fusion with the host cell membrane the viral
nucleocapsid is released into the host cell. When genes encoding
therapeutic agents are genetically engineered into the viral genome
15 under the control of promoters that are recognized by the host cell
(ie. baculovirus IE1 gene promoter), then this system can be utilized
for gene delivery purposes. The obvious example uses gp120 and the
AIDS virus. Engineering ODV to have a 23-CD4 receptor located on the
viral envelope, enables the targeting of that virus directly to HIV
20 infected cells. After membrane fusion with the HIV infected cell, a
gene that is under the control of an appropriate promoter encoding an
anti-HIV toxic protein could be delivered and expressed. The
resultant protein production would mediate the killing of the HIV
infected cell.
25   Baculovirus infection and DNA replication have been analyzed in a
number of species. These included: intraperitoneal injection of
virus into white mice (Ignoffo, 1971); feeding virus to rats, white
mice and guinea pigs (Ignoffo et al., 1972; Ignoffo and Heimpel,
1965) and inoculating mammalian cell lines with virus (Ignoffo and
30 Rafajko, 1972; Roder and Punter, 1977). Over the past 25 years, a
large body of literature has been collected that confirms the safety
of wild type baculoviruses. The overwhelming conclusion from this
literature with inoculation of a large number of hosts with

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Another use for such targeting and gene delivery system might include precise delivery of a gene to a cell type lacking a normal function. By placing the gene of interest in the baculovirus genome using transposable elements according to this invention, the gene,

5 upon transposition, becomes stably integrated into the genome of the target cell.

Signal targeting to the nuclear membrane into a vertebrate is also envisioned. This application takes into account several potential properties of the cell by any one of a number of viral vectors

10 (adenovirus, herpesvirus, retrovirus adeno-associate virus, etc.) or cell transformation systems to transform the cell for the novel targeting sequence, including constitutive expression and insertion of 23AA - X protein for whatever function desired.

If the nuclear membrane insertion signal functions as the data

15 shows, then the targeting sequence could be inserted into the nuclear envelope of any cell (vertebrate, invertebrate, or lower eukaryotes) by a variety of transformation or vector systems. An example might include a sindbis virus transformation vector carrying an insecticidal or lethal 23 - X protein into mosquito cells.

20   EXAMPLE 11
              APPLICATION OF THE 23 AMINO ACID TARGETING
                   SEQUENCE -- PROTEIN TARGETING

Data show that proteins containing the 23 amino acid sequence locate to the nuclear envelope, both the outer and inner nuclear

25 membrane, during defined times of the cell cycle. This location has been shown in both insect (Sf9) cell lines and mammalian (COS) cell lines during mitosis (as indicated by bi-nucleated cells). Thus, in actively dividing cells, the fusion protein is located in the nuclear membrane; while in non-dividing cells, the fusion protein is

30 incorporated within a general, but undefined set of cytoplasmic membranes. By using targeted ODV gene delivery, it becomes possible to specifically deliver a gene using the modified ODV envelope containing targeting proteins, express a gene encoding a drug or

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toxin and deliver that drug or toxin to the nuclear membrane of actively dividing cells. This approach provides another level of molecular approaches to potentially control or modify the organization and function of the nuclear envelope.

5   a.   Control of Cell Cycle
        Using either gene delivery or germline transformation approaches, it may be possible to target cells which are undergoing mitosis. An example might include selective targeting of a cancerous tissue growth. All of the cells of that tissue type contain the same
10   defining surface receptor, so normal and non-cancerous cell would be targets for the modified ODV. All cells would receive the gene encoding the therapeutic or toxic agent, however the therapeutic effects would only be active in dividing cells, when the protein located to the nuclear membrane. In this manner, cancerous cells,
15   which are dividing at an accelerated rate, would be primary recipients of the therapeutic agent or toxic agent.
        Examples of places in the cell mitotic event that may lend itself to interruption include: not allowing nuclear membrane dissolution (ie. through interrupting the lamin B phosphorylation pathway) or
20   adding a copy of cyclin B that has been genetically modified so that it cannot be ubiquinated and thus freezing the cell in metaphase (Hunter, 1993). These are two such examples, however there are many processes and temporal activities within the cell cycle that potentially could be altered or modified by foreign protein
25   localization to the nuclear envelope and specifically the inner nuclear membrane.

        b.   Delivery of Proteins to the Nuclear Membrane For Therapeutic Use
        By using protein targeting, germline transformation techniques
30   and/or gene delivery approaches, proteins could be delivered to the inner nuclear membrane that may function to interfere with or modify the effects of or functions of, known oncogenes. Both approaches, in above Examples 11(a) and 11(b), are potential uses of this technology

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to deliver proteins to the nuclear membrane of cancerous cells for cancer therapy. However, this approach should not be limited to cancerous conditions. Any malfunction in a cell that could be corrected or treated with gene/protein delivery to the nuclear

5   membrane would be a candidate for use of this technology.

                          EXAMPLE 12
          APPLICATION OF 23 AMINO ACID TARGETING SEQUENCE - -
                          DIAGNOSTICS

The instant invention can be used to produce a variety of

10 different proteins that locate to the ODV envelope. Using the 23 amino acid sequence of this invention would be especially beneficial for producing diagnostics based on receptors or integral membrane protein or protein complexes. Briefly, the advantages of this system are: (a) proteins can be placed in the ODV envelope in a known

15 orientation; (b) ODV can be generated in insect larva rather than the more expensive *in vitro* systems; and (c) the ability to incorporate ODV into viral occlusions at will allows ease of purification of viral occlusions and ODV, and ability to store the preparation for extended periods of time. Viral occlusions are easy to purify and

20 allow for long-term storage in that form.

The ability to produce certain diagnostic reagents in insect larva allows the protein reagent(s) to be produced more efficiently, inexpensively and with less effort compared with producing proteins from tissue culture systems. Fig. 11 is a diagram comparing the

25 levels of protein expression *in vitro* (tissue culture cells) with the levels in larva for several proteins (applicable to diagnostic applications). Protein can be produced *in vitro* and in insect larva utilizing recombinant baculovirus at high levels.

As can be seen for certain proteins, the expression level can be

30 much higher in larva than in tissue culture cells. For example, one insect larvae can provide enough material for approximately 30,000 ELISA diagnostic tests. Another advantage of isolating viral occlusions from larva (and not from tissue culture cells) is that the

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virus and incorporated proteins are extremely stable in the occlusion
form. Once viral occlusions have been purified, they can be stored
for years before final purification of ODV and sample use.

*   *   *

5   The chart shown on Fig. 13 outlines many of the potential
applications for the N-terminal 23 amino acid (23AA) sequence as
described in the preceding Examples.

*   *   *

Other embodiments are within the following claims.

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* * *

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of certain aspects of the present invention. Inclusion of reference
5 in this list is not intended to and does not constitute an admission
that the reference represents prior art with respect to the present
invention.

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- 87 -

What is claimed is:

1.   A substantially pure DNA encoding a transport polypeptide, the DNA comprising a strand which hybridizes at high stringency to a probe comprising a sequence of at least 15
5   consecutive nucleotides as shown in Fig. 1B, the polypeptide sharing at least 80% sequence identity with a series of corresponding amino acids listed in Fig. 1B.

2.   The DNA of claim 1, wherein the transport polypeptide is a polypeptide, 23AA, which directs localization of a desired protein
10 to occlusion derived virus envelopes.

3.   A vector comprising the DNA of claim 2.

4.   The DNA of claim 2, wherein the DNA is operably linked to regulatory sequences for expression of the protein, the regulatory sequences comprising a suitable promoter.

15   5.   A cell comprising the DNA of claim 4.

6.   An essentially homogeneous population of cells, each of which comprises the DNA of claim 4.

7.   A substantially pure transport polypeptide, wherein the polypeptide:

20   (a)   contains a hydrophobic domain; and
        (b)   localizes to occlusion derived virus envelopes thereby directing the localization of fused or hybrid proteins to occlusion derived virus envelopes.

8.   The polypeptide of claim 7, the polypeptide sharing at
25 least 80% sequence identity with the amino acids listed in Fig. 1B.

- 88 -

9.   An antibody which specifically binds to an epitope of the
polypeptide of claim 7, or an antigen-binding fragment of the
antibody.

10.   The antibody or antibody fragment of claim 9, wherein the
5 antibody or antibody fragment is linked to a detectable label.

11.   An antigenic fragment of a polypeptide having the amino
acids identified in Fig. 1B.

12.   A substantially pure DNA comprising a sequence of at
least 15 consecutive nucleotides of the region from nucleotides 122
10 to 190, inclusive, of Fig. 1A.

13.   The substantially pure DNA of claim 12 comprising a
sequence of at least 15 consecutive nucleotides of the region
encoding the transport polypeptide of Fig. 1A.

14.   A method of directing a desired protein to occlusion
15 derived virus envelopes comprising the steps of:
        (a)   constructing a vector comprising a marker protein and the
                DNA of claim 2;
        (b)   fusing a nucleotide sequence encoding a desired protein
                to the DNA of Step (a) to form a hybrid vector;
20   (c)   incubating the hybrid vector of Step (b) with a
                biological sample; and
        (d)   determining the localization of the marker protein within
                the biological sample, wherein localization indicates the
                presence of occlusion derived virus envelopes and also
25   delivery of the hybrid vector to the envelopes.

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15. A method of detecting 23AA transport polypeptide in a
biological sample comprising contacting the biological sample with
the antibody or antibody fragment of claim 9 and determining whether
the antibody or antibody fragment binds to a component of the sample,
5 the binding being an indication that the sample contains 23AA
transport polypeptide having a hydrophobic domain.

16. Isolated DNA having a nucleotide sequence selected from
the group consisting of:

     (a)   the nucleotide sequence of Fig. 1B; and
10   (b)   nucleotide sequences which, through the degeneracy of the
            genetic code, encode the same peptide gene product as
            that encoded by the nucleotide sequence of Fig. 1B.

1/14

```
1     CGGTGTTGGCGTCTATTTGTTTAAAACACTCGTTCGGCAAATGCGAGTGGTTGGACAAAAATATAAAAAC
                         DraI

71    TGTGTGTTTACAATTAAGAAAAATTTGTATCAATAATAAGCAACATTCGACATGTCTATCGTATTGATTA
1                                                        (ODV-E66) M  S  I  V  L  I  I
141   TTGTCATAGTTGTAATATTTTTAATATGTTTTTTGTACCTATCAAATAGCAATAATAAAAATGATGCCAA
8      V  I  V  V  I  F  L  I  C  F  L  Y  L  S  N  S  N  N  K  N  D  A  N
211   TAAAAACAATGCTTTTATTGATCTCAATCCCTTGCCGCTCAATGCTACAACCGCTACTACTACCACTGCC
31     K  N  N  A  F  I  D  L  N  P  L  P  L  N  A  T  T  A  T  T  T  T  A
281   GTTGCTACCACCACTACCAACAACAACAACAGCATAGTGGCCTTTCGGCAAAACAACATTCAAGAACTAC
54     V  A  T  T  T  T  N  N  N  N  S  I  V  A  F  R  Q  N  N  I  Q  E  L  Q
351   AAAACTTTGAACGATGGTTCAAAAATAATCTCTCATATTCGTTTAGCCAAAAAGCTGAAAAGGTGGTAAA
78       N  F  E  R  W  F  K  N  N  L  S  Y  S  F  S  Q  K  A  E  K  V  V  N
421   TCCCAATAGAAATTGGAACGACAACACGGTATTTGACAATTTGAGTCCGTGGACAAGCGTTCCGGACTTT
101    P  N  R  N  W  D  N  D  T  V  F  D  N  L  S  P  W  T  S  V  P  D  F
491   GGTACCGTGTGCCACACGCTCATAGGGTATTGCGTACGCTACAACAACACCAGCGACACGTTATACCAGA
124   G  T  V  C  H  T  L  I  G  Y  C  V  R  Y  N  N  T  S  D  T  L  Y  Q  N
         KpnI
561   ACCCTGAATTGGCTTACAATCTCATTAACGGGCTGCGCATCATTTGCAGCAAACTGCCCGATCCGCCGCC
148      P  E  L  A  Y  N  L  I  N  G  L  R  I  I  C  S  K  L  P  D  P  P  P
631   GCACCAACAAGCGCCCTGGGGCCCGGTCGCCGATTGGTACCATTTCACAATCACAATGCCCGAGGTGTTT
171    H  Q  Q  A  P  W  G  P  V  A  D  W  Y  H  F  T  I  T  M  P  E  V  F
                                        KpnI
701   ATGAACATTACCATTGTGCTAAACGAAACGCAGCATTACGACGAAGCTGCGTCCCTCACGCGTTACTGGC
194    M  N  I  T  I  V  L  N  E  T  Q  H  Y  D  E  A  A  S  L  T  R  Y  W  L
771   TCCGGCTTGTATCTGCCCACGGCCGTCAACTCGATGGGCTGGCACCGGACGGCAGGCAACTCAATGCGCAT
217      G  L  Y  L  P  T  A  V  N  S  M  G  W  H  R  T  A  G  N  S  M  R  M
841   GGGTGTGCCCTACACGTACAGTCAAATGTTGCGCGGATATTCATTGGCGCAAATTAGGCAAGAGCAGGGA
241    G  V  P  Y  T  Y  S  Q  M  L  R  G  Y  S  L  A  Q  I  R  Q  E  Q  G
911   ATACAAGAAATCCTAAACACGATCGCGTTTCCGTACGTGACTCAAGGCAACGGCTTGCACGTCGATTCGA
264    I  Q  E  I  L  N  T  I  A  F  P  Y  V  T  Q  G  N  G  L  H  V  D  S  I
981   TATACATCGATCACATTGACGTGCGCGCTTACGGCTATTTGATAAATTCATACTTTACGTTTGCCTATTA
288      Y  I  D  H  I  D  V  R  A  Y  G  Y  L  I  N  S  Y  F  T  F  A  Y  Y
1051  CACGTACTATTTTGGAGACGAGGTAATCAACACGTGGGTTTGACGAGAGCCATCGAAAACGTGGGCAGT
311    T  Y  Y  F  G  D  E  V  I  N  T  V  G  L  T  R  A  I  E  N  V  G  S
1121  CCCGAGGGAGTTGTGGTGCCAGGCGTCATGTCTCGAAACGGCACGTTGTACTCTAACGTGATAGGCAACT
334    P  E  G  V  V  V  P  G  V  M  S  R  N  G  T  L  Y  S  N  V  I  G  N  F
1191  TTATTACGTATCCGTTGGCCGTCCATTCGGCCGATTACTCCAAAGTGTTGACCAAACTTTCAAAAACATA
358      I  T  Y  P  L  A  V  H  S  A  D  Y  S  K  V  L  T  K  L  S  K  T  Y
1261  TTACGGTTCGGTTGTGGGCGTAACGAATAGGTTGGCTTACTACGAATCCGATCCCACAAACAACATTCAA
381    Y  G  S  V  V  G  V  T  N  R  L  A  Y  Y  E  S  D  P  T  N  N  I  Q
1331  GCGCCCCTGTGGACCATGGCGCGGCGCATTTGGAATCGGCGCGGCAGAATTATCAACTATAATGCCAACA
404    A  P  L  W  T  M  A  R  R  I  W  N  R  R  G  R  I  I  N  Y  N  A  N  T
1401  CGGTGTCGTTTGAGTCGGGTATTATTTTGCAAAGTTTGAACGGAATCATGCGCATCCCGTCGGGCACCAC
428      V  S  F  E  S  G  I  I  L  Q  S  L  N  G  I  M  R  I  P  S  G  T  T
1471  GTCCACGCAGTCGTTCAGACCGACCATTGGCCAAACGGCTATAGCCAAAACCGACACGGCCGGCGCCATT
451    S  T  G  S  F  R  P  T  I  G  Q  T  A  I  A  K  T  D  T  A  G  A  I
1541  TTGGTGTACGCCAAGTTTGCGGGAAATGAACAATTTGCAATTTAAATCGTGCACGTTGTTCTACGATCACG
474    L  V  Y  A  K  F  A  E  M  N  N  L  Q  F  K  S  C  T  L  F  Y  D  H  G
1611  GCATGTTCCAGCTATATTACAACATTGGCGTGGAACCAAACTCGCTCAACAACACAAACGGGCGGGTGAT
498    M  F  Q  L  Y  Y  N  I  G  V  E  P  N  S  L  N  N  T  N  G  R  V  I
1681  TGTGCTAAGCAGAGACACGTCGGTCAACACCAACGATTTGTCATTTGAAGCGCAAAGAATTAACAACAAC
521    V  L  S  R  D  T  S  V  N  T  N  D  L  S  F  E  A  Q  R  I  N  N  N
1751  AACTCGTCGGAAGGCACCACGTTCAACGGTGTGGTCTGTCATCGCGTTCCTATCACAAACATCAACGTGC
544    N  S  S  E  G  T  T  F  N  G  V  V  C  H  R  V  P  I  T  N  I  N  V  P
1821  CTTCTCTGACCGTTCGAAGTCCCAATTCTAGCGTCGAACTAGTCGAGCAGATAATTAGTTTTCAAACAAT
568    S  L  T  V  R  S  P  N  S  S  V  E  L  V  E  Q  I  I  S  F  Q  T  M
1891  GTACACGGCCACGGCTTCGGCCTGTTACAAATTAAACGTCGAAGGTCATTCGGATTCCCTGAGAGCTTTT
591    Y  T  A  T  A  S  A  C  Y  K  L  N  V  E  G  H  S  D  S  L  R  A  F
1961  AGAGTTAATTCCGACGAAAACATTTATGTAAACGTGGGCAACGGCGTTAAAGCCCTGTTTAATTATCCCT
614    R  V  N  S  D  E  N  I  Y  V  N  V  G  N  G  V  K  A  L  F  N  Y  P  W
2031  GGGTAATGGTCAAAGAAAATAACAAAGTGTCTTTCATGTCGGCTAACGAAGACACTACTATACCATTTAG
638      V  M  V  K  E  N  N  K  V  S  F  M  S  A  N  E  D  T  T  I  P  F  S
2101  CGTTATAATGAATTCCTTCACCTCTATCGGCGAACCAGCTTTGCAATACTCTCCATCAAATTGCTTTGTG
661    V  I  M  N  S  F  T  S  I  G  E  P  A  L  Q  Y  S  P  S  N  C  F  V
         EcoRI
2171  TATGGAAACGGTTTCAAATTGAACAACAGCACGTTTGATTTACAATTTATTTTTGAAATTGTGTAATTAT
684    Y  G  N  G  F  K  L  N  N  S  T  F  D  L  Q  F  I  F  E  I  V  *
2241  ATTTAGGGAGAATGTGATATTCAAAAGACTGACTGTTAACACAAAAGACTGATATTGTTGTTGTTACAAA
2311  ATAGATAATAAAACAAAAAATAAATTAAATATTATTTATTTATTAAACTGTTTAATTTTAATGCTAACGC
2381  GTACAAATCACGCTGTTCCGACGTGGACATGGAATTGCGCAGAAAAGTCTTGATAGTGTCGATTTCTTCG
2451  CCGTCATCCACTTCCATATATTTGATTTCTTCCTCGATTTGCATTTCCAAGTTTGCGTATTCTTGCAAAT
2521  AATAATCTAG
```

FIG 1a

Fig. 1B
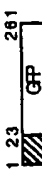
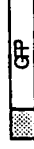**NUCLEOTIDE AND AMINO ACID SEQUENCE FOR 23AA:**

```
A T G   T C T   A T C   G T A   T T G   A T T   A T T   G T C
M       S       I       V       L       I       I       V

A T A   G T T   G T A   A T A   T T T   T T A   A T A   T G T
I       V       V       I       F       L       I       C

T T T   T T G   T A C   C T A   T C A   A A T   A G C
F       L       Y       L       S       N       S
```

AMINO ACID SEQUENCE FOR 23AA:

M S I V L I I V I V   V I F L I C F L Y L   S N S

Name	Structure	Promoter	Gene Locus	Production of Viral Occlusion	Protein Location
1. ODV-E66	(1 23 ... 125 704)	ODV-E66	ODV-E66	Yes	ODV-Env, M, NE
2. 125β-gal	(1 23 ... 125 ... 1140 β-gal)	ODV-E66	ODV-E66	Yes	ODV-Env, M, NE
3. Δ2-23β-gal	(β-gal)	ODV-E66	ODV-E66	Yes	—
4. 23β-gal	(1 23 β-gal)	ODV-E66	ODV-E66	Yes	ODV-Env, M, NE
5. pAcUW21-23GFP	(1 23 ... 261 GFP)	p10	polyhedrin	Yes	ODV-Env, M, NE, CM
6. pVL1393-23GFP	(GFP)	polyhedrin	polyhedrin	No	ODV-Env, M, NE, CM
7. pVL1393-E66F	(1 23 ... 125 704)	polyhedrin	polyhedrin	No	ODV-Env, M, NE, CM
8. pAcUW21-23URF13	(1 23 ... 138 URF13)	p10	polyhedrin	Yes	ODV-Env, M, NE
9. pIE1HR4-23GFP (uninfected Sf9 cells)	(GFP)	IE1	N/A	N/A	Cell cycle regulation to NE
10. pCMV-23GFP (uninfected COS-1 cells)	(GFP)	CMV-IE	N/A	N/A	Cell cycle regulation to NE
11. pRTL2-23GFP (Plant cells)	(GFP)	35S	N/A	N/A	?
12. pVL1392-24GFP (ODV-E25)	(GFP)	polyhedrin	polyhedrin	?	?

ODV-Env - ODV envelope
M - Intranuclear microvesicles and membrane structures
NE - Nuclear envelope
CM - Cytoplasmic membranes
CMV-IE - Major immediate early promoter of cytomegalovirus
N/A - Not applicable

▨ N-terminal 23 amino acid hydrophobic sequence derived from ODV-E66

▦ N-terminal 24 amino acid hydrophobic sequence derived from ODV-E25

FIG 3

FIG 3a

FIG 3b

0.2 μm

0.5 μm

0.5 μm
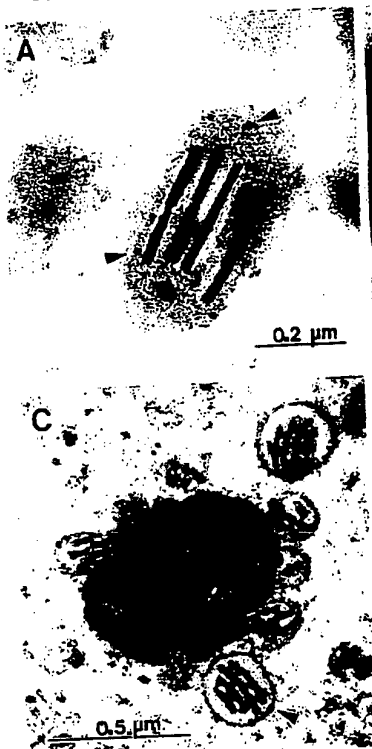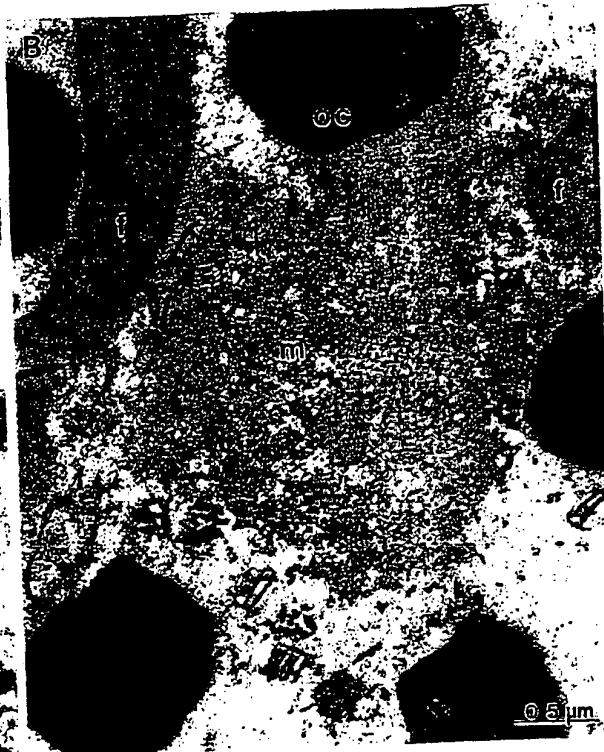
FIG 3c

FIG 4a

FIG 4b

FIG 4c

FIG 5a

FIG 5b

FIG 6a   FIG 6b   FIG 6f

FIG 6g

FIG 6e

FIG 7a

FIG 7b

FIG 8a   FIG 8c   FIG 8e
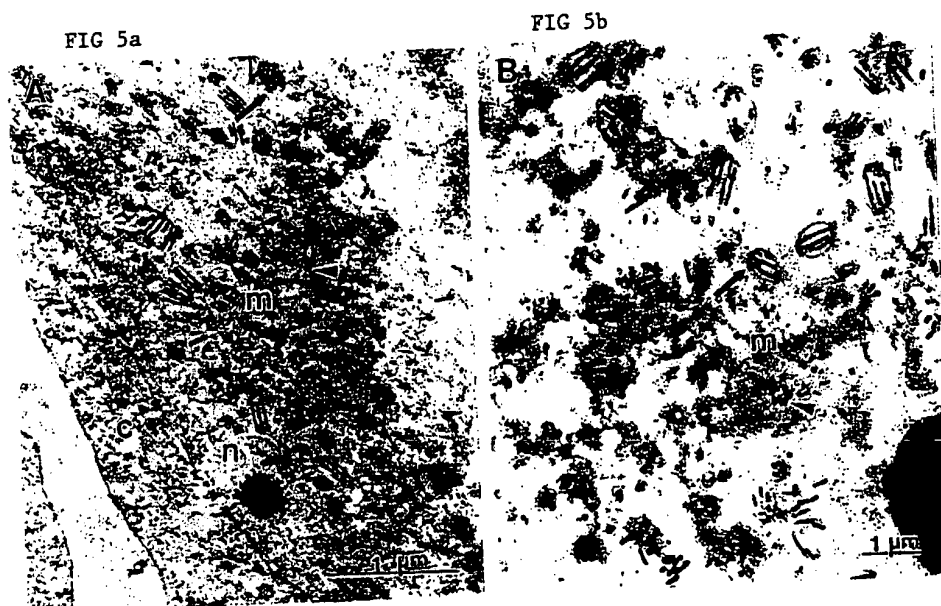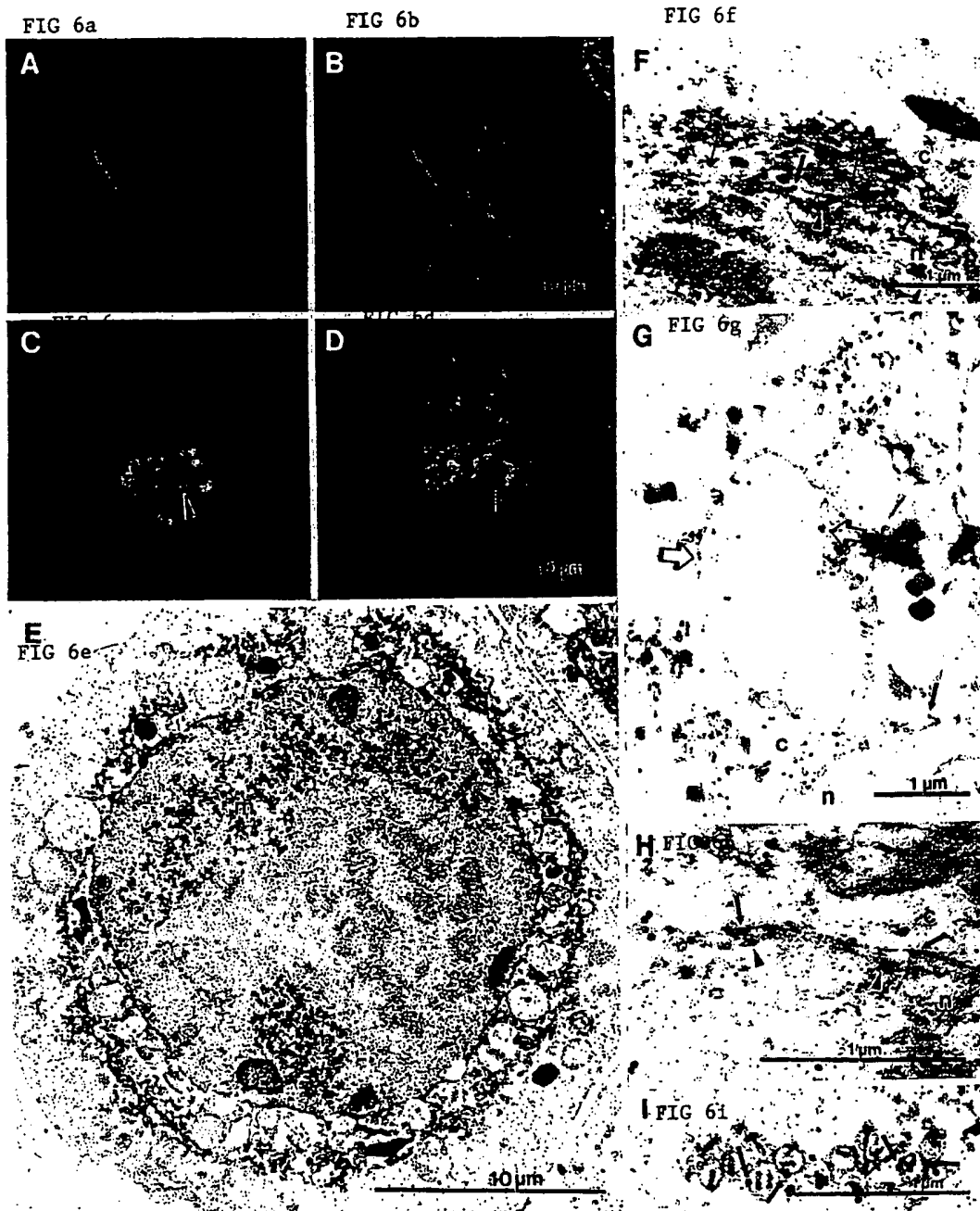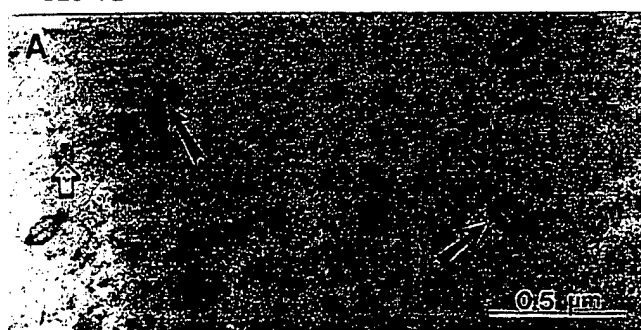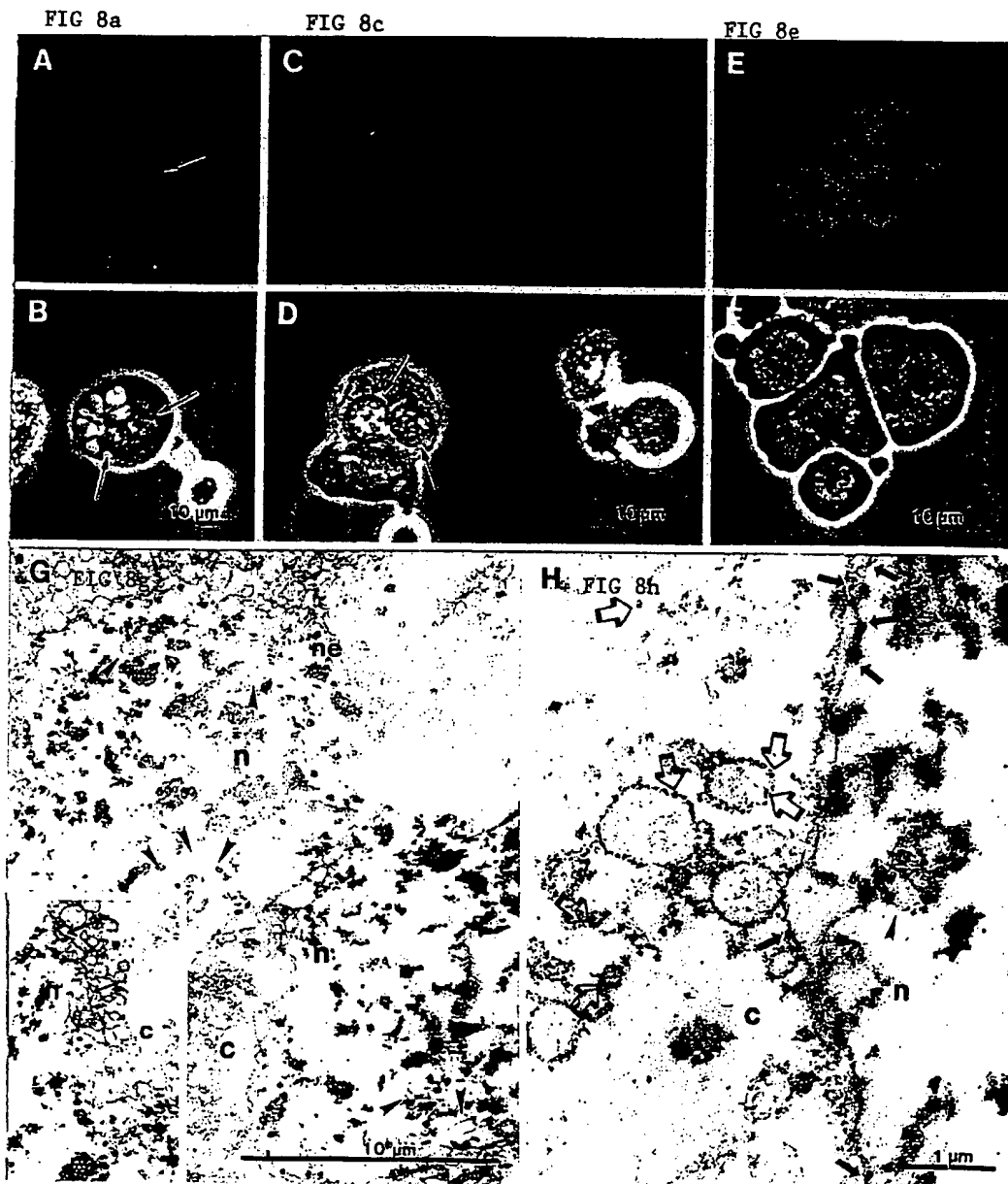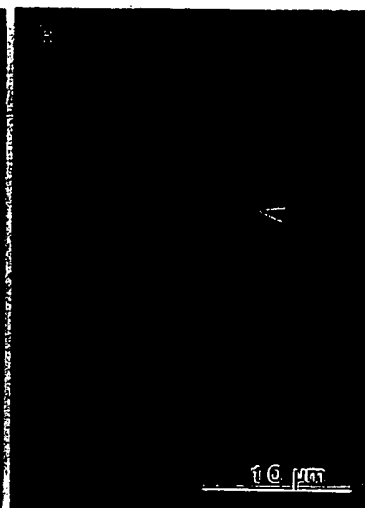
FIG 9a
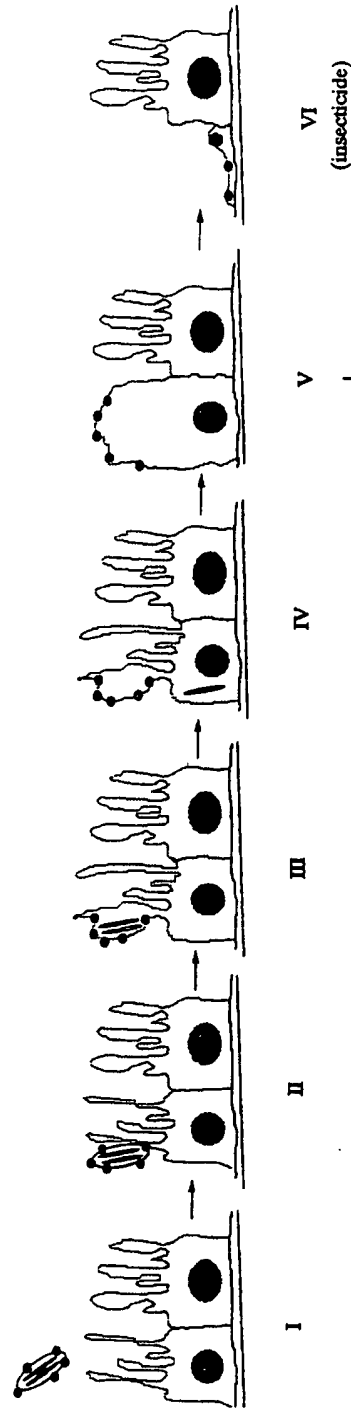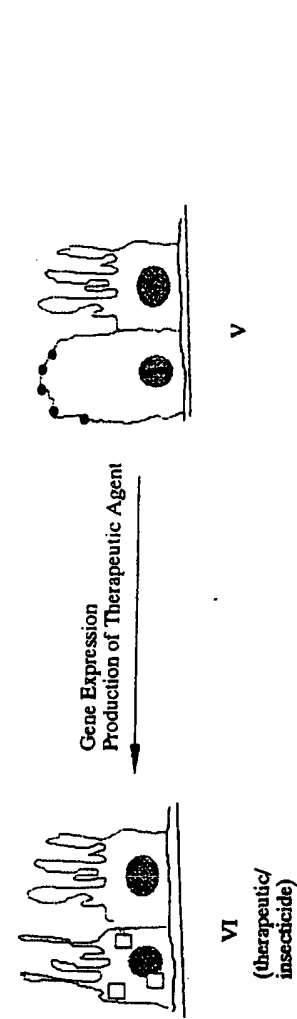
FIG 9b

A. Directed Delivery to Target Cell

FIG 10

B. Directed Delivery to Target Cell with Subsequent Gene/Drug Delivery

FIG 11
LARVAL-TISSUE CULTURE EXPRESSION LEVELS

	Tissue Culture	Larval	References
Firefly luciferase	*S. littoralis* >25% total protein	*T. ni* 15% total protein	Jha *et al.*, 1990
IgG2A	*T. ni* 6.4 µg/ml supernatant	*T. ni* 800 µg/larva/7 dpi	Reis *et al.*, 1992
Murine retinoic acid receptor	Sf9 cells 300 pmol/mg protein	*Manduca sexta* 100 pmol/mg protein	Ross *et al.*, 1992
VSV-N protein	Sf9 cells 41-60% total protein	*Spodoptera exigua* 68% total protein larva = 30,000 ELISAS	Ahmad *et al.*, 1993
Rinderpest virus N protein		*Spodoptera frugiperda* larva = 15,000 sera tests	Ismail *et al.*, 1994
Insulin-like growth factor II		silkworm larva 3.6 mg/larva	Marumoto *et al.*, 1987
Human α interferon		silkworm larva 50 µg/hemolymph/larva	Maeda *et al.*, 1985
Bacterial luciferase		*E. acrea* 195 µg/larva	Richardson *et al.*, 1992
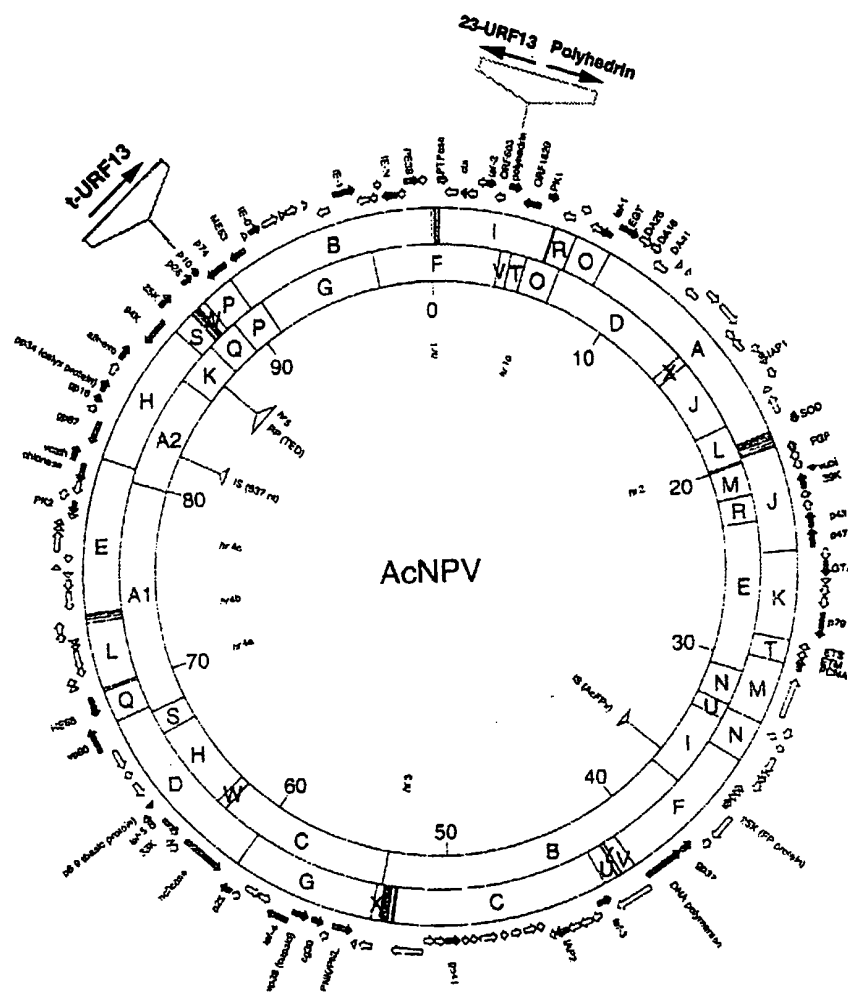
Genomic map of *Autographa californica* NPV. Multiple genes can be inserted at both the polyhedrin and p10 locus. In this manner, recombinant virus can be constructed that are occlusion +, and contain both 23-URF13 and WT t-URF13. (Ayres *et al.*, 1994)

Development of Fusion-Protein Construct (23 a.a.)

PCR, codon useage, codon degenerancy,
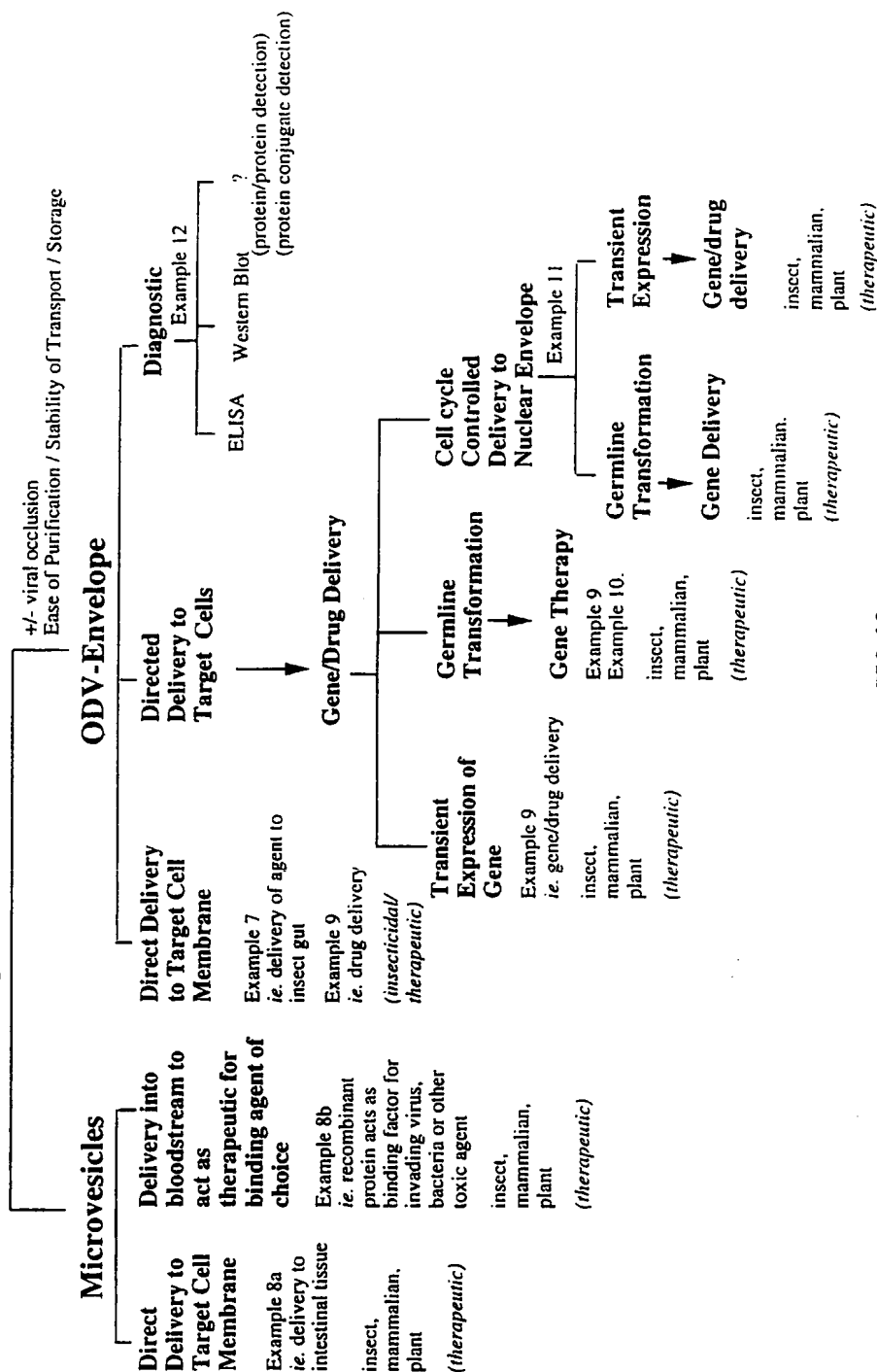functional or structural cognates, etc.

Development of Recombinant Baculovirus

+/- viral occlusion
Ease of Purification / Stability of Transport / Storage

ODV-Envelope

Microvesicles

Direct Delivery to Target Cell Membrane	Delivery into bloodstream to act as therapeutic for binding agent of choice	Direct Delivery to Target Cell Membrane	Directed Delivery to Target Cells	Diagnostic

Direct Delivery to Target Cell Membrane

Example 8a
ie. delivery to intestinal tissue

insect,
mammalian,
plant

(therapeutic)

Delivery into bloodstream to act as therapeutic for binding agent of choice

Example 8b
ie. recombinant protein acts as binding factor for invading virus, bacteria or other toxic agent

insect,
mammalian,
plant

(therapeutic)

Direct Delivery to Target Cell Membrane

Example 7
ie. delivery of agent to insect gut

Example 9
ie. drug delivery

(insecticidal/ therapeutic)

Directed Delivery to Target Cells

Gene/Drug Delivery

Transient Expression of Gene

Example 9
ie. gene/drug delivery

insect,
mammalian,
plant

(therapeutic)

Germline Transformation

Gene Therapy

Example 9
Example 10.

insect,
mammalian,
plant

(therapeutic)

Cell cycle Controlled Delivery to Nuclear Envelope

Example 11

Germline Transformation

Gene Delivery

insect,
mammalian,
plant

(therapeutic)

Transient Expression

Gene/drug delivery

insect,
mammalian,
plant

(therapeutic)

Diagnostic

Example 12

ELISA   Western Blot ?
(protein/protein detection)
(protein conjugate detection)

FIG 12